UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

El receptor cannabinoide CB_2R como diana terapéutica y herramienta pronóstico/predictiva en cáncer de mama HER2+

CB_2R cannanoid receptor as therapeutic target and prognostic/predictive tool in HER2+brest cancer

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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EL RECEPTOR CANNABINOIDE CB_2R COMO DIANA TERAPÉUTICA Y HERRAMIENTA PRONÓSTICO/PREDICTIVA EN CÁNCER DE MAMA HER2+

CB_2R CANNABINOID RECEPTOR AS THERAPEUTIC TARGET AND PROGNOSTIC/PREDICTIVE TOOL IN HER2+ BREAST CANCER

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A mi abuelo Lili,
por haber estado siempre tan orgulloso de mi
aunque no entendieras una palabra de lo que hacía...
...y porque te echo de menos cada día.

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Manolín, rey de GhanaTogoBenin! Creo que no se puede admirar a alguien más. Tener la suerte de trabajar contigo y aprender de ti todos estos años, ha sido un lujo y un regalo. Dejas una huella en cada uno de nosotros, por tu calidad científica, pero sobre todo por tu calidad humana. Me has aportado unas herramientas y una visión de afrontar la vida que me llevo conmigo para siempre!

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RESUMEN

El cáncer de mama es una de las enfermedades más frecuentes en todo el mundo y representa un importante problema de salud pública. Se trata de una enfermedad muy heterogénea que se clasifica en distintos subtipos atendiendo a marcadores moleculares. Uno de ellos se caracteriza por la sobreexpresión del receptor del factor de crecimiento epidérmico 2 (HER2), y representa un 20%-25% de todos los cánceres de mama diagnosticados. Aunque estos tumores son muy agresivos, el pronóstico de las pacientes ha mejorado enormemente con el desarrollo de terapias dirigidas contra este receptor. Sin embargo, las tasas de resistencia innata y adquirida son muy elevadas y, por tanto, sigue siendo necesaria la búsqueda de tanto nuevos tratamientos para estas pacientes, como de herramientas que permitan la identificación temprana de aquellas con alto riesgo de no responder a terapias estándar o de recaer.

El Δ^9-tetrahidrocannabinol (THC), principal compuesto activo de la planta *Cannabis sativa* L., produce efectos antitumorales en distintos modelos de cáncer, incluido el de mama HER2+, a través de la activación de receptores cannabinoides. Estos receptores, pertenecientes a la superfamilia de receptores acoplados a proteínas G y denominados CB1 y CB2, junto con sus ligandos endógenos y las enzimas encargadas de la síntesis y degradación de estos últimos, constituyen el llamado sistema endocannabinoide (SEC). El SEC ha emergido como un sistema indispensable de comunicación celular que regula gran variedad de funciones biológicas. Además, se han encontrado alteraciones de sus distintos componentes en multitud de enfermedades, entre las que cabe destacar el cáncer. Por ejemplo, en cáncer de mama, se ha descrito la sobreexpresión del receptor CB2 (CB2R) en tejido tumoral frente a la mama sana. Aunque distintos trabajos señalan a este receptor como diana antitumoral en este tipo de tumores, no hay apenas información sobre su papel en la fisiopatología del cáncer de mama.

En este contexto, el primer objetivo de la presente Tesis Doctoral fue esclarecer el papel de CB2R en la generación y progresión del cáncer de mama. En primer lugar, encontramos una elevada expresión de este receptor en tumores del tipo HER2+, que además se asociaba con un peor pronóstico de las pacientes. Para estudiar si los niveles anormalmente altos de CB2R contribuían de alguna manera a la agresividad de estos tumores, analizamos el efecto de su eliminación en un modelo genético de cáncer de mama HER2+ (el ratón MMTV-neu). Observamos que los ratones MMTV neu: CB2R $^{-/-}$ desarrollan tumores más tarde que sus hermanos MMTV neu: CB2R WT y que una vez que aparecen, su crecimiento es significativamente más lento. Por otro lado, la sobreexpresión de CB2R en distintas lineas de cáncer de mama produjo un aumento de sus propiedades pro-oncogénicas a través de la activación de la señalización promovida por HER2. Concretamente, observamos que HER2 regula al alza la expresión de CB2R a través de la activación del factor de transcripción ELK1 vía ERK, y que estos elevados niveles de CB2R favorecen la señalización oncogénica de HER2 a través de la proteína tirosina quinasa no receptora c-SRC. Todos estos datos sugieren que CB2R desempeña un papel protumoral en el cáncer de mama HER2+.

Esta fuerte asociación entre HER2 y CB2R dio lugar al planteamiento del segundo objetivo de esta Tesis: la caracterización exhaustiva de una posible interacción física entre HER2 y CB2R en cáncer de mama HER2+. A través de distintas técnicas experimentales, demostramos la existencia de heterómeros HER2-CB2R tanto en cultivos celulares como en tumores humanos HER2+. Analizamos además su expresión en dos microarrays de tejidos que contenían casi un centenar de distintas muestras humanas y encontramos una asociación significativa entre una alta expresión de estos complejos y una menor supervivencia libre de enfermedad, y una menor supervivencia global, lo que sugiere que los heterómeros HER2-CB2R se asocian con un peor pronóstico de las pacientes.

Finalmente, en el tercer objetivo de la presente Tesis, nos planteamos evaluar si los heterómeros HER2-CB2R podrían ser considerados una nueva diana terapéutica en cáncer de mama HER2+. Primero observamos que el tratamiento con THC, a través de la activación selectiva de CB2R, promueve la rotura de los heterómeros, lo que conduce a la inactivación de HER2 a través de la separación de los dímeros HER2-HER2, y a su degradación a través del proteasoma vía la E3 ligasa c-CBL. Todo esto, desencadena respuestas antitumorales tanto *in vitro* como *in vivo*. Además, conseguimos demostrar que el segmento 5 de CB2R está implicado en la interacción física con HER2, y que utilizando herramientas específicamente dirigidas a bloquear estéricamente esta interacción producían efectos similares a los del THC. Todo ello sugiere que la rotura de los heterómeros HER2-CB2R podría ser una nueva estrategia terapéutica para el tratamiento del cáncer de mama HER2+.

En resumen, los resultados obtenidos en esta Tesis revelan el papel protumoral del receptor CB2 en cáncer de mama HER2+, y ponen de manifiesto un nuevo mecanismo de regulación de la actividad oncogénica de HER2 a través de la heterodimerización con CB2R. Además, estos resultados muestran a los heterómeros HER2-CB2R como nuevas dianas antitumorales y biomarcadores con valor pronóstico en cáncer de mama HER2+.

ABSTRACT

Breast cancer is one of the most frequent malignancies worldwide and represents an important public health problem. This disease is very heterogeneous and is subclassified in different subtypes according to molecular markers. One of them is characterized by the overexpression of the human epidermal growth factor receptor 2 (HER2), and represents 20%–25% of all breast carcinomas. Although these tumors are very aggressive, the clinical outcome of HER2+ breast cancer patients has greatly improved with the development of targeted therapies against this receptor. However the innate and acquired resistance rates to these therapies are still significant and therefore, new therapies are urgently warranted for these patients as well as new tools to identify those at a higher risk of not responding or recurring.

Δ^9-Tetrahydrocannabinol (THC), the main bioactive component of the plant *Cannabis sativa* L., has been shown to exert antitumor actions in different models of cancer, including HER2+ breast cancer, by activating cannabinoid receptors. These receptors, which belong to the G protein-coupled receptor superfamily and are known as CB1R and CB2R, together with their endogenous ligands and the enzymes responsible for their synthesis and degradation, constitute the so-called endocannabinoid system (ECS). The ECS has emerged as an indispensable cell-communication system, involved in a plethora of biological functions. Moreover, alterations in different components of the ECS have been reported in multiple pathologies, including cancer. In the context of breast cancer specifically, previous works show an overexpression of CB2R in tumoral tissue as compared to the healthy mammary epithelium. Although strong evidence point to this receptor as a target for anticancer therapy, there is no information about its role in the physiopathology of breast cancer.

In this context, the first aim of this Doctoral Thesis was to unravel the role of CB2R in breast cancer tumorigenesis. First, we found that CB2R is highly expressed in HER2+ tumors, and that this elevated expression correlates with poor patient prognosis. To determine whether high CB2R expression favors HER2+ tumor aggressiveness, we analyzed the effect of its genetic ablation on a genetic model of breast cancer: the MMTV-neu mouse. We found that MMTV-neu: CB2R$^{-/-}$ mice had a delay in tumor onset and a decrease in tumor growth as compared with their WT littermates. Moreover, we observed that CB2R overexpression confers pro-oncogenic properties on breast cancer cells by activating HER2-driven signaling. Specifically, we observed that HER2 upregulates CB2R expression by activating the transcription factor ELK1 *via* the ERK cascade, and that increased CB2R expression activates HER2 pro-oncogenic signaling at the level of the non-receptor tyrosine kinase c-SRC. Altogether, these data suggest that CB2R plays a protumoral role in HER2+ breast cancer.

The strong association between HER2 and CB2R led to the second aim of this Thesis: the exhaustive characterization of a potential physical interaction between HER2 and CB2R in HER2+ breast cancer. By using a wide variety of techniques, we cogently demonstrate the existence of HER2-CB2R heteromers in cell cultures as well as in human HER2+ tumors. We also analyzed their expression in two different tissue microarrays containing rougly 100 HER2+ human breast cancer samples.

We found a significant correlation between high heteromer expression and lower disease-free and overall survival, suggesting that HER2-CB2R heteromers are associated to poor patient prognosis.

Finally, in the third aim of this Thesis, our goal was determining whether HER2-CB2R heteromers could be considered new targets for the manegement of HER2+ breast cancer. We found that THC disrupts HER2-CB2R complexes by selectively binding to CB2R, which leads to the inactivation of HER2 through disruption of HER2-HER2 homodimers, and its degradation by the proteasome *via* the E3 ligase c-CBL. This, in turn, triggers antitumor responses *in vitro* and *in vivo*. Moreover, we were able to demonstrate that CB2R transmembrane segment 5 is involved in the physical interaction with HER2, and that using tools specifically designed to sterically hamper this intercation led to THC-like effects. Together, these findings suggest that disruption of HER2-CB2R complexes might be a new therapeutic strategy for the treatment of HER2+ breast cancer.

In summary, the results obtained in this Thesis reveal that, CB2R plays a protumoral role in HER2+ breast cancer, and unveil a new mechanism controlling the oncogenic activity of HER2, its heteromerization with CB2R. Moreover, these findings define HER2-CB2R heteromers as new potential targets for antitumor therapies and biomarkers with prognostic value in HER2+ breast cancer.

ABBREVIATIONS

2-AG: 2-arachidonoylglycerol

5-HT: 5-hydroxytryptamine

AC: adenylyl cyclase

ADAM: a disintegrin and metalloproteinase

AEA: arachidonoylethanolamide

AREG: amphiregulin

ATP: adenosine triphosphate

BiFC: bimolecular fluorescence complementation

BRET: bioluminescence resonance energy transfer

BTC: betacellulin

CB_{1R}: cannabinoid receptor type-1

CB_{2R}: cannabinoid receptor type-2

CBD: cannabidiol

CBG: cannabigerol

CBL: casitas B-lineage lymphoma

CBN: cannabinol

CBR: cannabinoid receptor

CDK: cyclin-dependent kinase

CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

ChIP: chromatin immunoprecipitation

CHIP: COOH-terminus of HSP70-interacting protein

CNS: central nervous system

COX2: cyclooxygenase-2

CXCR4: C-X-C chemokine receptor type 4

CYP: cytochrome P450 protein

DAB: 3, 3 -diaminobenzidine

DAG: diacylglycerol

DAGL: diacylglycerol lipase

DAPI: 4’,6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl-sulfoxide

eCB: endocannabinoid

ECS: endocannabinoid system

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ELK1: ETS domain-containing protein 1

ERK: extracellular regulated-signal kinase

EPR: epiregulin

ER: estrogen receptor

ERK: extracellular signal-regulated kinase

FAAH: fatty acid amide hydrolase

FBS: fetal bovine serum

FDA: food and drug administration

FGR: feline gardner-rasheed sarcoma viral oncogene homolog

FISH: fluorescent in situ hybridization

FRET: Förster resonance energy transfer

FYN: tyrosine-protein kinase Fyn

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GFP: green fluorescent protein

GPCR: G protein coupled-receptor

GPR55: G protein-coupled receptor 55

GRB2: growth factor receptor-bound protein 2

GRP18: glycine-rich protein 18

GUSB: glucuronidase beta

HB-EGF: heparin-binding EGF-like growth factor

HCK: hematopoietic cell kinase

HEK: human embryonic kidney

HER: human epidermal growth factor receptor

HSP90: heat shock protein 90

ID-1: inhibitor of DNA binding-1

IGFR: insulin-like growth factor 1 receptor

IHC: immunohistochemistry

LCK: lymphocyte-specific protein tyrosine kinase

LC-MS: liquid chromatography–mass spectrometry

LOX: lipoxygenase

LYN: LCK/YES novel tyrosine kinase

MAGL: monoacylglycerol lipase

MAPK: mitogen-activated protein kinase

MASP: marker-assisted selection protocol

MMP: matrix metalloproteinase

MMTV: mouse mammary tumor virus

mTOR: mammalian target of rapamycin complex

MUC-4: membrane-associated glycoprotein mucin-4

NAPE: N-acylphosphatidylethanolamine

NAPE-PLD: N-acylphosphatidylethanolamine-hydrolyzing phospholipase D

NAT: N-acyltransferase

NEM: N-ethylmaleimide

NRG: neuregulin

OR: opioid receptor

PCR: polymerase chain reaction

PDX: patient-derived xenograft

PEI: polyethylenimine

PFA: paraformaldehyde

PI3K: phosphatidylinositol-3-kinase

PKA: protein kinase A

PLA: proximity ligation assay

PLCɣ: phospholipase C gamma

PMSF: phenylmethylsulfonyl fluoride

PPAR: peroxisome proliferator-activated receptor

PR: progesterone receptor

PTB: polypyrimidine-tract-binding protein

PTEN: phosphatase and tensin homolog protein

PVDF: polyvinylidene difluoride

RhoA: Ras homolog family member A

RIPA: radioimmunoprecipitation assay

ROS: reactive oxygen species

RTK: receptor tyrosine kinase

S1P: sphingosine-1-phosphate

SCID: severe combined immunodeficiency

SH2: Src Homology 2

SHC1: SH2 domain protein C1

SNP: single nucleotide polymorphism

STR: short term repetitions

TAT: transactivator of transcription

TBS: Tris-buffered saline

T-DM1: Trastuzumab-DM1

TGFα: transforming growth factor-α

THC: Δ^9-tetrahydrocannabinol

TIMP-1: tissue inhibitor of matrix metalloproteinases-1

TM: transmembrane

TMA: tissue microarray

TRIB3: pseudo-kinase tribbles homolog 3

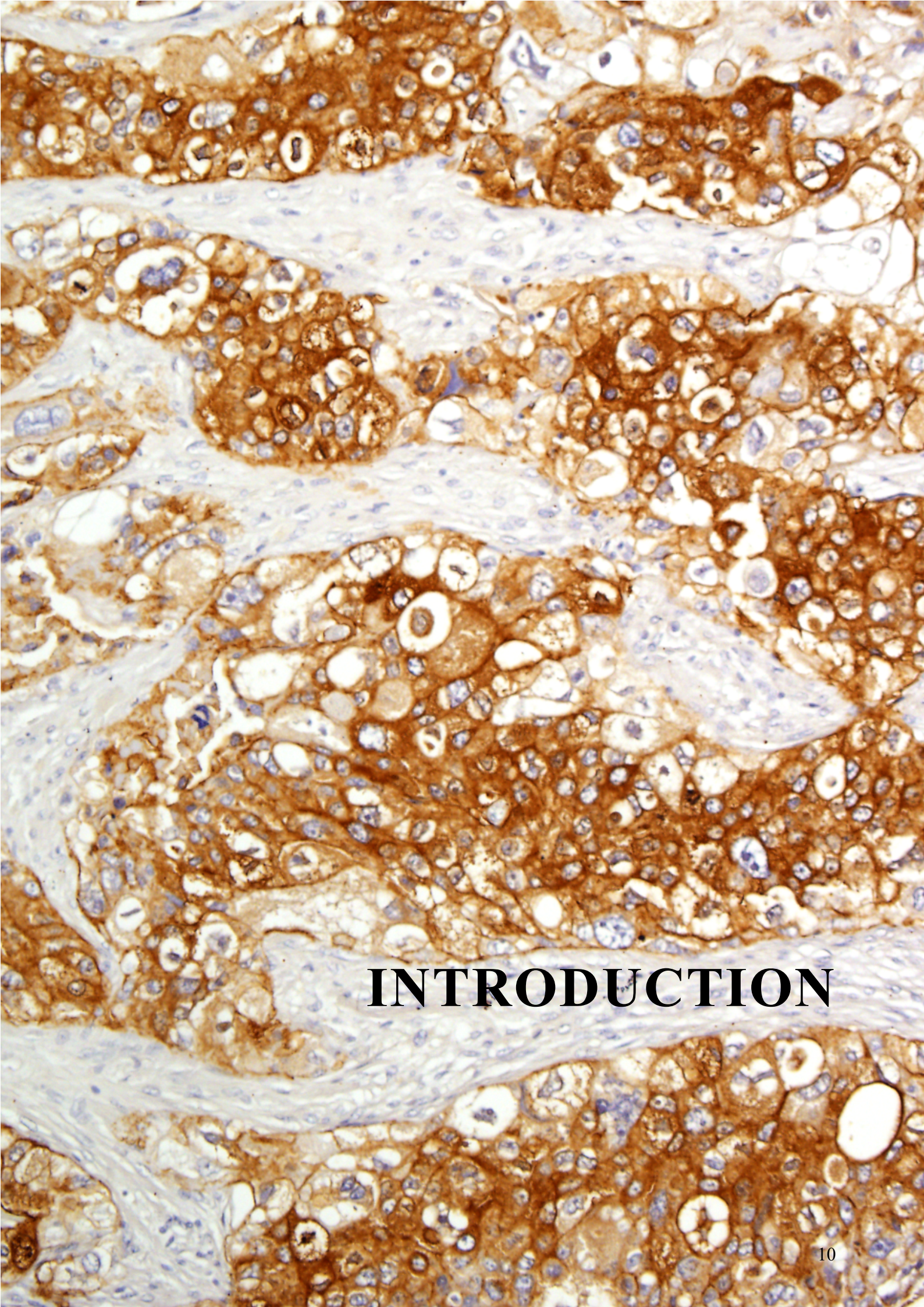
TRPV1: transient receptor potential cation channel subfamily V, type 1

Tyr: tyrosine

VEGF: vascular endothelial growth factor

YES: YES proto-oncogene 1, Src family tyrosine kinase

YFP: yellow fluorescent protein



INTRODUCTION

1. BREAST CANCER

Breast cancer is the second most common cancer in the world, and by far the most frequent among women, with an estimated 1,67 million new cases diagnosed in 2012 (25% of all cancers) (Ferlay *et al.* 2015), which means that one out of eight women will get breast cancer during their lifetime. It is also the most frequent cause of cancer death in women from less developed regions (324.000 deaths in 2012) and the second in women from more developed countries (198.000 deaths in 2012). From a clinical, genetic and phenotypic point of view, breast cancer is a highly heterogeneous disease (Hergueta-Redondo *et al.* 2008). Thus, breast cancer cannot be viewed as a single clinico-pathological entity, but it must be necessarily dissected into more homogeneous and clinically relevant subtypes that help to predict prognosis and therapeutic responses, leading to different clinical strategies.

1.1. BREAST CANCER CLASSIFICATION

Historically, breast cancer has been classified according to the location and aggressiveness of the disease. To understand the classical histopathological classification, it is necessary to briefly introduce the anatomo-functional organization of the mammary gland (Figure 1). This organ is comprised of a tubulo-glandular system embedded in stromal connective tissue, and the mammary fat pad that extends from the nipple towards the tubules. The functional portion of the mammary gland is the ductal lobular unit, and this structure is formed by two cell layers: an epithelial one surrounding the central lumen (luminal cells), and a myoepithelial layer, in contact with the basement membrane (basal cells), which separates the epithelium from the stroma. Thus, the classical histological classification of breast cancer distinguishes two main subgroups depending on whether the tumor originates within the ducts that transport milk to the nipples (ductal carcinoma) or in the lobes that secrete milk to the ducts (lobular carcinoma).

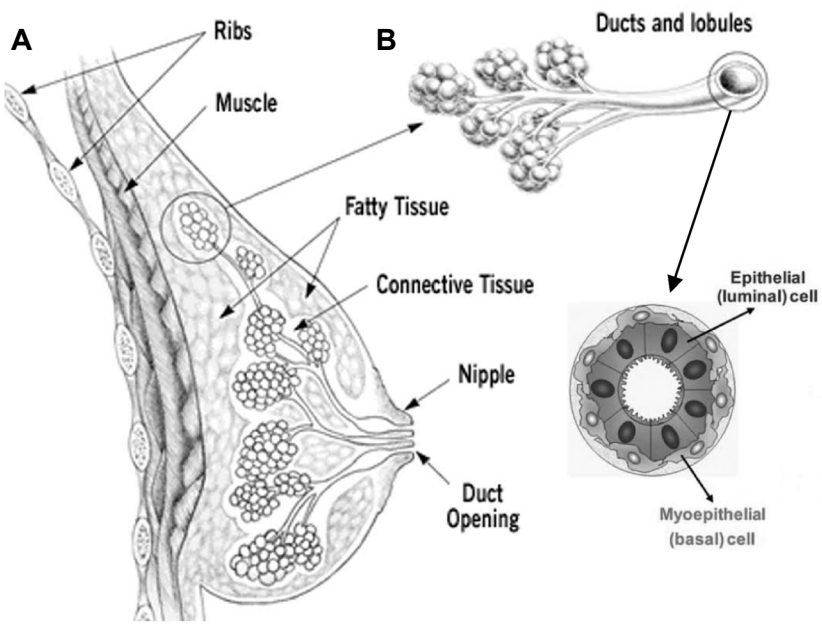


Figure 1. Anatomy of the mammary gland. (A) Scheme of the mammary gland anatomy. The breast is composed of glandular and stromal tissue. Glandular tissue includes the ducts and lobules. **(B)** Insight in the terminal duct-lobular unit, which is the basic functional unit of the breast. **(C)** Cellular components of a mammary duct: inner layer of epithelial (luminal) cells and the outer layer of myoepithelial (basal) cells. Adapted from Hergueta-Redondo *et al.* 2008.

Moreover, breast cancer is classified as *in situ* carcinoma when the tumor has not spread, or invasive carcinoma when the tumor cells have invaded the surrounding breast tissue (Malhotra *et al.* 2010). However, this histopathological classification is insufficient in terms of prognosis and predictive implications, and its clinical utility is quite modest (Viale 2012). Therefore, additional features are also evaluated in order to better characterize and treat this disease. The assessment of the histological grade is a routine analysis that gives valuable prognostic information. According to the Elston and Ellis criteria (Elston 1985), breast cancer can be subdivided into three tumoral differentiation grades: Grade I (well differentiated), Grade II (moderately differentiated) and Grade III (poorly differentiated), based on the relative presence of well-defined tubules/glands, nuclear atypia and mitosis number. In addition, breast cancer has also been classified according to molecular phenotypes based on the expression of markers with high predictive value: estrogen and progesterone receptors (ER and PR, respectively), and overexpression/amplification of the epidermal growth factor receptor 2 (HER2) (Figure 2). These molecular markers have historically defined three breast cancer subtypes in terms not only of their molecular features but also their potential responsiveness to targeted treatments: hormone-sensitive (ER+/PR+), whose therapeutic approach is to shut down the estrogenic signaling; HER2+, which are treated with anti-HER2 selective drugs; and triple-negative, which due to the lack of specific markers, receive indiscriminate chemotherapy (Higgins and Baselga 2011). The epidemiology and treatment of these breast cancer subtypes will be discussed in more detail in the following sections.

Taken together, ER, PR and HER2 expression, in conjunction with other clinical and pathological factors such as age, menopausal status, tumor size, histological grade and lymphovascular invasion, have been classically used and are still used for breast cancer patient prognosis and management (Dai *et al.* 2015). Over the last decades, high-throughput microarray and other "omics" analyses such as genomic, transcriptomic or proteomic approaches, have been applied to the study of breast cancer biology in order to better characterize and classify this heterogeneous disease. The pioneer studies conducted by Perou´s group reported distinctive molecular signatures for 5 different subtypes of breast cancer by analyzing 65 surgical breast tumors specimens from 42 different patients using complementary DNAs microarrays. Thus, based on gene expression profiling, these studies (Perou *et al.* 2000; Sorlie *et al.* 2001) demonstrated the existence of at least five different breast cancer phenotypes: Luminal-like A and B, HER2-positive, Basal-like and Normal-like (Figure 2).

Luminal tumors: Luminal-like tumors are the most common subtype among breast cancer (64%). They are characterized by the expression of hormone receptors and of other genes typically present in epithelial cells. These tumors have the best prognosis of all breast cancer subtypes and can be divided in two different subtypes: luminal A, which have higher expression of ER-related genes and lower expression of proliferative genes, and luminal B, which tend to be of higher grade than luminal A tumors. A percentage of the latter overexpress HER2 as well.

HER2-enriched tumors: This subtype is defined by the overexpression of HER2 and the lack of ER and PR expression, accounts for approximately 15% of all invasive breast cancers, and is associated to poor patient prognosis. Given that this Thesis will focus on HER2-positive breast cancer, it will be extensively introduced in the next section.

Basal-like tumors: This group lacks expression of ER, PR and HER2, and is characterized by high expression of basal markers such as citokeratins 5, 6, 14, 17 and EGFR. It represents approximately 15% of the invasive breast cancers and is the most aggressive subtype.

Normal-like tumors: It represents approximately 8% of all breast cancer subtypes and it is poorly characterized. These tumors lack expression of HER2, ER and PR and present a genetic signature characteristic of normal adipose tissue.

More recently, a new breast cancer subtype was identified and termed **claudin-low** (Herschkowitz *et al.* 2007). These tumors are characterized by low / absent expression of luminal differentiation markers, high enrichment of epithelial to mesenchymal transition markers and cancer stem cell-like features.

Molecular sub-classification of breast cancer is a field in constant growth and will probably set the bases for better and more effective personalized treatments. However, and despite the growing number of clinically relevant molecular subtypes being identified, current breast cancer patient management still depends on traditional pathology assessment and the expression of classical predictive markers (ER, PR, HER2), complemented in some cases with genetic signature tests (*i.e.*, MammaPrint, MapQuant Dx and Oncotype DX, Theros).

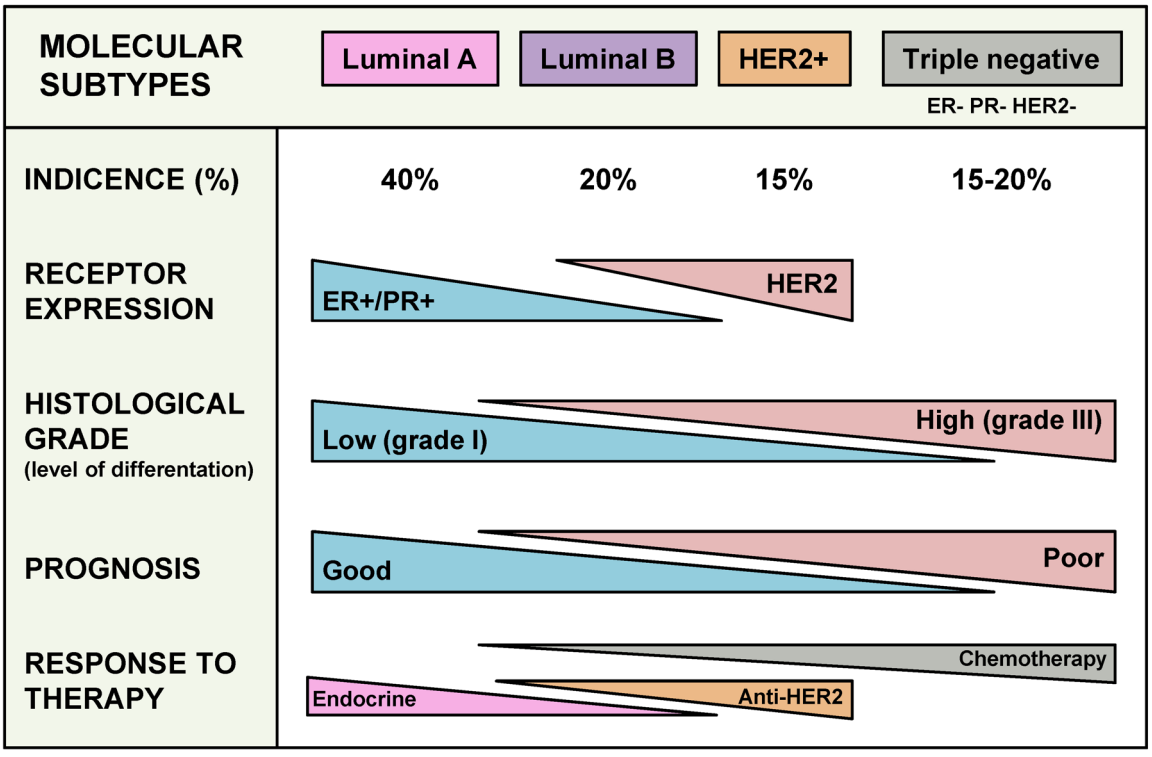


Figure 2. Schematic representation of the main breast cancer subtypes identified in the analysis of gene expression profiling: Luminal A (ER+, PR+, HER2-), Luminal B (ER+, PR+. HER2+), HER2+ and Triple negative (ER-, PR-, HER2-). Their most relevant characteristics are shown in the scheme.

1.2. HER2-POSITIVE BREAST CANCER

The human epidermal growth factor receptor 2 (HER2), also known as NEU or ERBB2, is a proto-oncogen mapped to chromosome 17q21, that encodes a 185 KDa transmembrane glycoprotein with tyrosine kinase activity (Schechter *et al.* 1984; Akiyama *et al.* 1986). The various naming systems for HER2 are the result of independent studies discovering homologous genes at the same time. Thus, the *neu* oncogene was initially described as a transforming oncogene discovered in a carcinogen induced rat brain tumor model (Shih *et al.* 1981), and was found homologous to the gene involved in the pathogenesis of erythroblastoma in chickens (*v-erbB*) (Vennström and Bishop 1982) and the human epidermal growth factor receptor (*EGFR*). Few years later, independent studies identified the *v-erbB* related gene in the human genome, similar but distinct from the *EGFR* gene, and one group named it *c-ERBB-2* (Semba *et al.* 1985), and the other termed it *HER2* (Coussens *et al.* 1985). Subsequent sequence analysis and chromosomal mapping studies revealed all three genes to be the same. To unify, we will call it *HER2* throughout this Thesis.

HER2 belongs to the epidermal growth factor receptor family, together with other three members, commonly referred as EGFR (HER1, ERBB1), HER3 (ERBB3), and HER4 (ERBB4) (Figure 3). These receptors are located at the plasma membrane, and are composed of an extracellular ligand binding domain, an α-helical transmembrane domain and an intracellular tyrosine kinase domain. HER receptors control key processes in the cells such as proliferation, differentiation, migration, and apoptosis. They also play an important role during development, since null mutations of any of the *ErbB* genes result in embryonic or perinatal lethality (Lee *et al.* 1995; Sibilia and Wagner 1995).

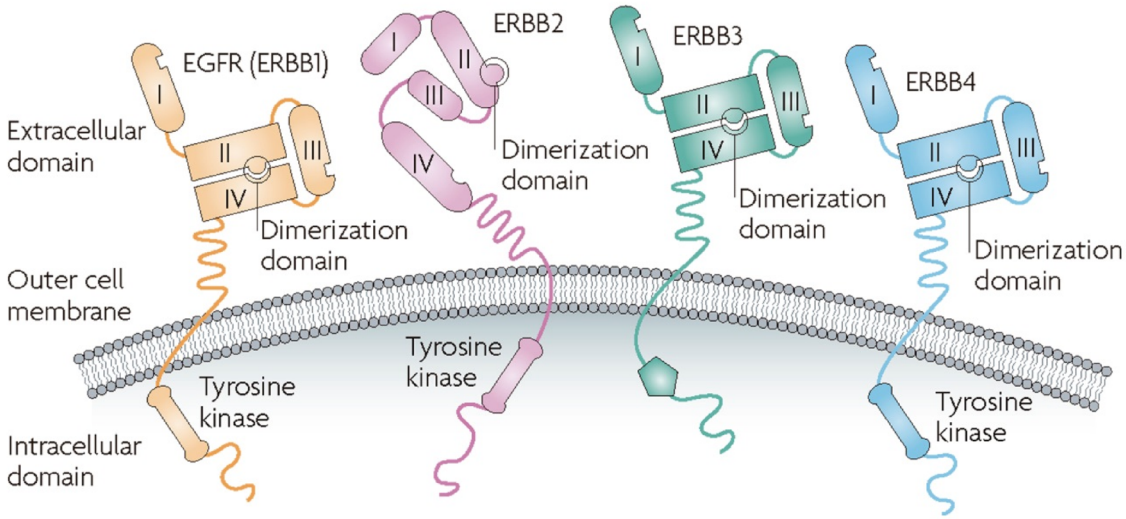


Figure 3. ERBB family members. The ERBB family consists of four members : ERBB1 (EGFR), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). Each receptor is composed of three functional domains: an extracellular domain responsible for ligand binding; the α-helical transmembrane segment; and the intracellular protein tyrosine kinase domain that also contains motifs and residues that mediate interactions with intracellular signalling molecules. EGFR, HER3 and HER4 exist in a "closed" conformation in which the dimerization domain is not available to interact with partner ERBB moieties in the absence of ligand. On the contrary, HER2 (which has no known ligand) exists in an active "open" conformation and is permanently available for dimerization. From Baselga and Swain 2009.

The importance of this receptor family and the cell functions it controls is further supported by the fact that their alteration is associated to different pathologies. For example, under physiological conditions, HER2 is involved in the regulation of normal breast growth and development (Jones and Stern 1999). However, an abnormal amplification of this receptor is associated with breast cancer development. The first evidence came in 1987 when Slamon *et al.* found that amplification of the HER2 gene was a significant predictor of both overall survival and time-to-relapse in patients with breast cancer (Slamon *et al.* 1987). HER2 is overexpressed in 20-30% of human breast cancers, and this is mainly due to gene amplification (generation of more than the normal two gene copies) and overexpression (Hynes 1994). Specifically, HER2+ breast cancers can have up to 25-50 copies of the HER2 gene and up to 40-100 fold increase in HER2 protein expression, resulting in up to 2 million receptor molecules expressed at the tumor cell surface. HER2 amplification and overexpression has also been observed in subsets of gastric, esophageal, endometrial, and ovarian cancer, and this feature is also associated with poor patient prognosis (Moasser 2007). Tumors are classed as HER2-positive if they show immunohistochemistry (IHC) staining of 3+, a fluorescent *in situ* hybridization (FISH) result of more than 6 HER2 gene copies per nucleus, or a FISH ratio of more than 2.2 (HER2 gene signals / chromosome 17 signals) (Wolff *et al.* 2006). This consensus and standardization of the criteria to define the HER2 positivity of a tumor has become a key element to distinguish the patients that could benefit from HER2-directed therapy from those who are unlikely to respond. The development of targeted therapies against HER2 has a tremendous therapeutic potential. For this reason, a deep understanding of HER2 biology is crucial to generate new pharmacological tools to target it.

1.2.1. HER2 activation

HER receptors can be activated by several ligands. Some of them bind exclusively to HER1, such as epidermal growth factor (EGF), transforming growth factor-α (TGFα) and amphiregulin (AREG), or bind exclusively to HER4, such as neuregulin 3 and 4 (NRG3 and NRG4). Others have a dual specificity and bind either both HER1 and HER4, such as betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF) and epiregulin (EPR), or bind both HER3 and HER4, such as neuregulin 1 and 2 (NRG1 and NRG2) [reviewed in (Olayioye *et al.* 2000)]. Despite the large number of ligands identified so far for HER1, HER3 and HER4, no ligand has been discovered for HER2 (see below). However, HER2 acts as the preferred partner for heterodimerization, participating in signal transduction in the absence of a specific ligand (Graus-Porta *et al.* 1997).

Ligand binding to the extracellular domain of HER receptors induces a conformational change from the inactive to the active state. Specifically, ligands bind to domains I and III, and promote a conformational change of the extracellular domain, exposing domain II, which facilitates receptor dimerization (Figure 4). Additional receptor interactions in extracellular, transmembrane and kinase domains further stabilize the dimerization of the receptors, an essential step for its subsequent activation. Unlike the other members of the family, the extracellular domain of HER2 does not pivot between active and inactive conformations

and constitutively exists in an activated conformation (Garrett *et al.* 2003). This unique feature explains why 1) HER2 has the strongest catalytic kinase activity of the HER2 family, 2) HER2-containing heterodimers are the most common and with the strongest signaling functions, and 3) HER2 has no endogenous ligand. Aside from heterodimers with other HER members, HER2 can form ligand-independent homodimers in HER2+ tumors which are constitutively active (Yarden 2001). This spontaneous dimer formation is apparently a consequence of the high density of HER2 receptors present in the cell membrane. Although HER2 homodimers alone may contribute to malignancy, it is well known that HER2 heterodimers play an important role in breast tumorigenesis. In fact, it has been described that the HER2:HER3 heterodimer is the most powerful oncogenic combination (Holbro *et al.* 2003; Dey *et al.* 2015).

Receptor dimerization promotes partial activation of their intrinsic tyrosine kinase activity, which triggers transphosphorylation of their intracellular domains followed by autophosphorylation of the same receptor region. The phosphorylated Tyr residues serve as docking sites for SH2- and PTB-containing adaptor proteins, which in turn recruit and activate proteins involved in the control of cell functions such as proliferation and survival (Figure 4). This is common for all the family members, except for HER3, which is catalytically inactive (Sierke *et al.* 1997). Consistent with this, the signaling functions of HER3 are mediated entirely through the kinase activity of its heterodimeric partners. Each HER receptor displays a distinct C-terminal phosphorylation pattern that provides different docking sites, and therefore activates different signaling pathways such phosphatidylinositol-3-kinase (PI3K) /Akt, Ras/extracellular signal-regulated kinase (Ras/ERK) or phospholipase Cɣ (PLCɣ) pathways (Baselga and Swain 2009) (Figure 4). For example, due to the presence of multiple binding sites for p85 (*i.e.* the main regulatory subunit of PI3K), HER3 is the most effective activator of PI3K/Akt pathway, whereas HER1 and HER2 are mainly coupled to Ras/ERK-dependent pathway, due to the presence of multiple sites for Shc and Grb2 (Hynes NE 1994).

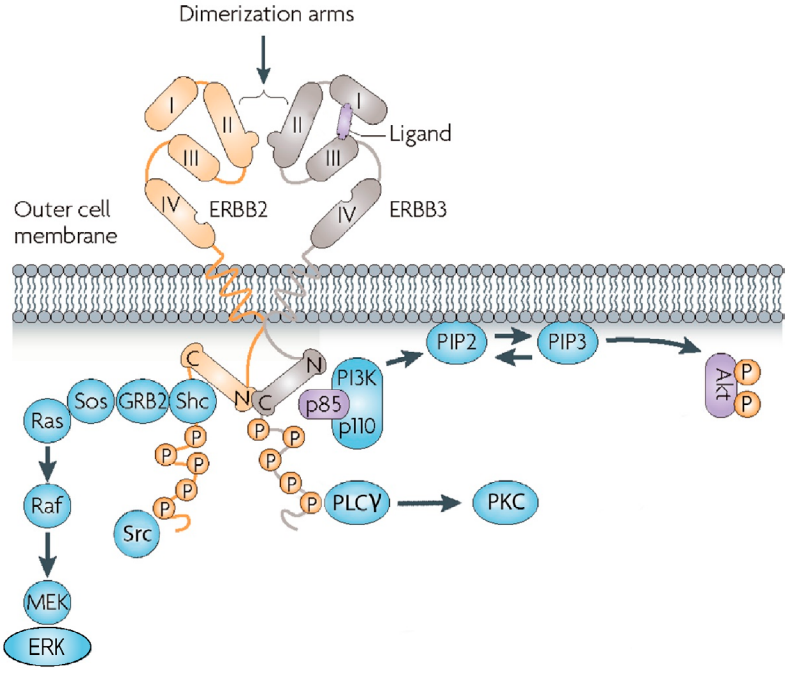


Figure 4. Signaling downstream of HER dimer formation. Ligand binding induces a conformational change that exposes the dimerization domain. Dimer formation results in the cross- and auto-phosphorylation of the dimer partners, creating docking sites that allow the recruitment of downstream signalling components and the formation of signalling complexes. The main signaling pathways triggered by ERBB dimers are: PI3K/Akt, Ras/ERK, phospholipase Cɣ and SRC kinases pathways, which in turn promotes cancer cell proliferation, survival, invasion, etc. Adapted from Baselga and Swain 2009.

1.2.2. HER2 degradation

As for other membrane receptors, an important mechanism of HER regulation involves the control of its degradation. The most studied HER receptor in terms of internalization and degradation is HER1. Upon ligand binding, HER1 is internalized and, once in the endosome, the ligand is separated from the receptor, and HER1 either recycles to the cell surface or is transported with the ligand to the lysosome, where it is degraded (Sorkin and Goh 2009). On the contrary, HER2 is highly resistant to internalization and degradation, and remains at the cell surface to signal during prolonged periods of time after activation. In addition, HER2-containing dimers undergo slower dissociation and internalization, and are more frequently recycled back to the cell surface for reactivation rather than targeted for degradation at the lysosome (Wang *et al.* 1999). Although the mechanisms underlying these different receptor dynamics are poorly understood, the ability to avoid internalization and to maintain signaling at the plasma membrane contributes to HER2 oncogenic capacity (Bertelsen and Stang 2014). As a possible explanation, it has been suggested that the C-terminal regulatory domain of HER2 either contains the molecular signals responsible for its retention at the cell surface or lacks the signals necessary to support the internalization, or that the conformation of the C-terminus may block the access to internalization (Bertelsen and Stang 2014). In addition, it has been shown that the evolutionary conserved chaperone HSP90 protects HER2 from misfolding and degradation (Xu *et al.* 2001). Supporting this observation, inhibition of HSP90 induces rapid HER2 polyubiquitination (Mimnaugh *et al.* 1996) by the E3-ligase CHIP (COOH-terminus of HSP70-interacting protein), followed by receptor down-regulation (Xu *et al.* 2002 Marx 2010). Moreover, other stimuli, such as anti-HER2 antibodies, have been reported to induce HER2 ubiquitination and degradation. Specifically, trastuzumab (an anti-HER2 antibody that will be discussed in more detail below) has been shown to enhance recruitment of the E3-ubiquitin ligase c-CBL to HER2, and to accelerate HER2 internalization and degradation (Klapper *et al.* 2000).

1.3. HER2- TARGETED THERAPIES

The discovery of HER2 overexpression in a significant percentage of breast cancer patients led to the development of HER2-targeted agents that have greatly improved the way HER2+ breast cancer patients are treated (Loibl and Gianni 2017). Different strategies have been followed to target HER2, such as anti-HER2 antibodies, inhibitors of HER2 dimerization, tyrosine kinase inhibitors, antibody-based conjugates and HSP90 inhibitors (Figure 5).

1.3.1. Monoclonal antibodies

Trastuzumab

Trastuzumab, the first anti-HER2 therapy ever developed (Carter *et al.* 1992), is a humanized monoclonal antibody directed against the extracellular domain IV of HER2 (Figure 5A). It is currently the first-line treatment for patients with metastatic HER2+ tumors, either as a single agent or in

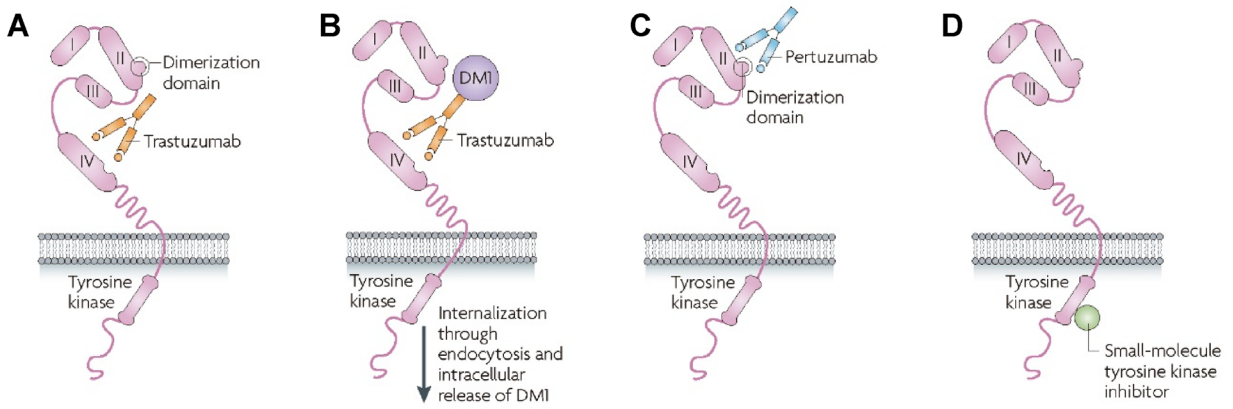


Figure 5. Current approaches to target HER2. (A) The antibody trastuzumab binds directly to domain IV of the extracellular region of HER2, blocking its signaling activity. **(B)** Trastuzumab-DM1 (T-DM1) consists of trastuzumab conjugated to the anti-microtubule agente DM1. After binding to HER2, T-DM1 is internalized and DM1 is released into the cancer cell to exert its cytotoxic effects. **(C)** The antibody pertuzumab binds to domain II of the extracelular region of HER2, preventing its dimerization, a key step in HER2 activation. These three antobodies exert their antitumoral actions by induction of antibody-dependent cellular cytotoxicity as well. **(D)** Inhibition of HER2 tyrosine kinase activity with small molecule inhibitors such as lapatinib. Adapted from Baselga and Swain 2009.

combination with endocrine therapy or chemotherapy (Awada *et al.* 2012). Although the mechanism of action of trastuzumab is not yet fully understood, it is likely to exert its antitumor activity through a combination of antibody-dependent cellular cytotoxicity (Clynes *et al.* 2000), antiangiogenic effects, downregulation of HER2 receptors (Klapper *et al.* 2000), disruption of downstream proliferative pathways (Junttila *et al.* 2009; Nagata *et al.* 2004), and inhibition of cell-cycle progression (Baselga *et al.* 2001). Even though trastuzumab has significantly improved the clinical outcome of HER2+ breast cancer patients, up to 40% of them do not respond to this treatment. In addition, about 70% of the patients that initially respond, eventually progress to metastatic disease within a year, indicating that secondary or acquired resistance to trastuzumab is not unusual (Vu and Claret 2012). Several mechanisms have been proposed as responsible for innate and acquired trastuzumab resistance:

1) Switch to other RTKs signaling. For example, inhibition of HER2-mediated activation of PI3K by trastuzumab may be bypassed by activation of HER3, thus promoting cellular proliferation (Holbro *et al.* 2003).

2) Resistance may be mediated through an altered interaction between the receptor and the antibody, either through mutations in the extracellular domain of HER2 that hamper binding of trastuzumab, the masking of HER2 antigens on the surface of tumor cells through overexpression of glycoproteins such as membrane-associated glycoprotein mucin-4 (MUC-4) (Price-Schiavi *et al.* 2002), or the overexpression of p95, a truncated form of HER2 that lacks its extracellular domain and thus does not have a trastuzumab binding site (Scaltriti *et al.* 2007; Arribas *et al.* 2011).

3) Alterations in HER2 downstream signaling, notably loss-of-function mutations in the phosphatase and tensin homolog protein (PTEN)(Nagata *et al.* 2004; Zhang *et al.* 2011), or activating mutations in PI3K (Berns *et al.* 2007) that lead to enhanced Akt phosphorylation and signaling, among others.

Current strategies to overcome trastuzumab resistance include combination with other HER2-targeted and/or HER2-independent treatments, which are increasing the magnitude and duration of the therapies responses.

Pertuzumab

Pertuzumab is a humanized monoclonal antibody targeting the portion of the extracellular domain of HER2 involved in receptor dimerization (Figure 5C). Specifically, it binds to an epitope in the subdomain II, which is distinct from the domain targeted by trastuzumab (Franklin *et al.* 2004). By preventing HER2 dimerization with other ligand-activated HER receptors, most notably HER3, pertuzumab inhibits the downstream activation of the PI3K and ERK pathways. Since pertuzumab and trastuzumab bind to different HER2 epitopes and have complementary mechanisms of action, the combination of these two agents produces a greater antitumor activity than either agent alone in preclinical models (Scheuer *et al.* 2001) and in clinical trials (Baselga *et al.* 2012; Perez *et al.* 2017), which led to the approval of their combination and docetaxel as first line treatment for HER2+ metastatic breast cancer (Pondé *et al.* 2018).

Trastuzumab-emtansine (T-DM1)

Trastuzumab-DM1 (T-DM1) is a novel antibody-drug conjugate, composed of a potent cytotoxic drug (emtansine) stably linked to trastuzumab (Figure 5B). By using this approach, HER2 is targeted and chemotherapy is delivered simultaneously only to cells overexpressing HER2, thus minimizing toxicity on non-tumor cells (Peddi and Hurvitz 2013). Specifically, T-DM1 binds to HER2 and subsequently enters the cell *via* endocytosis. T-DM1 then undergoes intralysosomal proteolytic degradation, resulting in the intracellular release of the cytotoxic agent and subsequent cancer cell death (Lewis Phillips *et al.* 2008). Currently, T-DM1 is given to HER2+ breast cancer patients as a second-line treatment (Pondé *et al.* 2018).

1.3.2. Tyrone kinase inhibitors

Lapatinib

Lapatinib is a small molecule dual (HER1 and HER2) tyrosine kinase inhibitor (Figure 5D). It reversibly binds to the ATP-binding site of HER2 and EGFR, preventing signal transduction of both Ras/ERK and PI3K/Akt pathways and, therefore, inhibiting proliferation and survival (Xia *et al.* 2002). Unlike trastuzumab, and since it targets the receptor´s intracellular domain, it can also inhibit the truncated form of HER2 (Scaltriti *et al.* 2007), which makes it a potential tool to overcome trastuzumab resistance mediated by p95. In addition, in preclinical models of HER2+ breast cancer, lapatinib produces synergistic antitumor effects when combined with trastuzumab, suggesting that these two agents could be combined in the clinical setting (Scaltriti *et al.* 2009). In fact, and although initially lapatinib was recomended as a second-line treatment, the last results of CLEOPATRA, EMILIA, and TH3RESA clinical trials (Krop *et al.* 2014; Diéras *et al.* 2017; Perez *et al.* 2017) have led to the inclusion of lapatinib in

combination with trastuzumab as a third-line treatment (Larionov 2018).

1.3.3. Therapies under development

HER2+ breast cancer is an excellent example of how scientific knowledge can change the outcome of patients. Twenty years ago, HER2+ breast cancer was considered an unfavorable diagnosis associated with poor patient prognosis. Understanding how this receptor works (how it is activated, the importance of the dimerization process, the key role of its kinase domain activity, etc.) led to the development of HER2-targeted therapies that have dramatically improved patients´ outcomes. Nowadays, a HER2+ breast cancer diagnosis is considered favorable, with about 70% of these patients reaching 10 year disease-free survival with the current HER2-targeted treatments (Larionov 2018). Nevertheless, metastatic HER2+ breast cancer is still an incurable disease and new therapeutic options are needed (Loibl and Gianni 2017). Some of the approaches that are being currently followed to tackle this and other remaining clinical challenges, such as resistance, include: (1) new tyrosine kinase inhibitors such as Neratinib, a pan-HER inhibitor (Awada *et al.* 2016), (2) inhibitors of HER2 downstream signaling such as PI3K and mTOR inhibitors (Dey and Leyland 2017), (3) inhibitors of HSP90, which have been shown to induce proteasomal degradation of HER2, cell cycle arrest and apoptosis in preclinical models of HER2+ breast cancer (Xu *et al.* 2001), (4) inhibitors of the cell cycle (*e.g.* CDK4/6 inhibitors) (Goel *et al.* 2016), (5) inhibitors of angiogenesis (*e.g.* bevacizumab, a VEGF inhibitor) (Manso *et al.* 2015), etc.

The design of new tools will be clearly favored by expanding our knowledge on how the activity of this receptor is modulated, including not only the activation process *per se*, but other crucial aspects in the control of its activity such as its deactivation and its degradation.

2. CANNABIS AND THE ENDOCANNABINOID SYSTEM

The hemp plant *Cannabis sativa* L. (commonly known as cannabis or marijuana) and its many different preparations have been used for millennia for recreational, medical or even religious purposes (Mechoulam *et al.* 2014). The first known record of the use of cannabis as a medicine comes from China 5000 years ago, and later, its use spread into India and other Asian countries, the Middle East, Asia, South Africa and South America (Hanuš and Mechoulam 2005). In Western Europe, cannabis was introduced during the 19th century by the Napoleonic soldiers returning from Egypt and by British physicians returning from India (Mechoulam and Parker 2013).

Research on the chemistry of cannabis began in the late-19th century (Figure 6), following a major trend in chemical research at that time, focused on the quest for active natural products. Numerous alkaloids, as for example morphine, cocaine and strychnine, were isolated from various plants, purified and used in medicine (Mechoulam and Hanuš 2000). However, more than a century passed until such advances were achieved in the cannabis research field. The lack of success to isolate the pure form and elucidate the structure of the main psychoactive constituents of marijuana was mainly

due to technical limitations (Mechoulam and Parker 2013). We currently know that cannabinoids are present in the plant as a mixture of many closely related constituents -over 100 - which were difficult to separate using the methods that were available in the 19th and early 20th centuries. Nonetheless, substantial progress was made during that time by the groups of Cahn and Todd in the United Kingdom, and Adams in the United States, which resulted in the isolation of the first plant cannabinoid, named cannabinol (CBN) (Mechoulam *et al.* 2014). However, it was not until 1964, with the advance of modern separation and analysis techniques, that the isolation and structure elucidation of the major psychoactive ingredient of marijuana, Δ^9-tetrahydrocannabinol (THC), was finally achieved (Gaoni and Mechoulam 1964). Shortly after, it was synthesized and became widely available for research (Mechoulam and Gaoni 1967). During those years, the same group was also successful in the isolation and structure characterization of the main nonpsychoactive plant cannabinoid: cannabidiol (CBD).

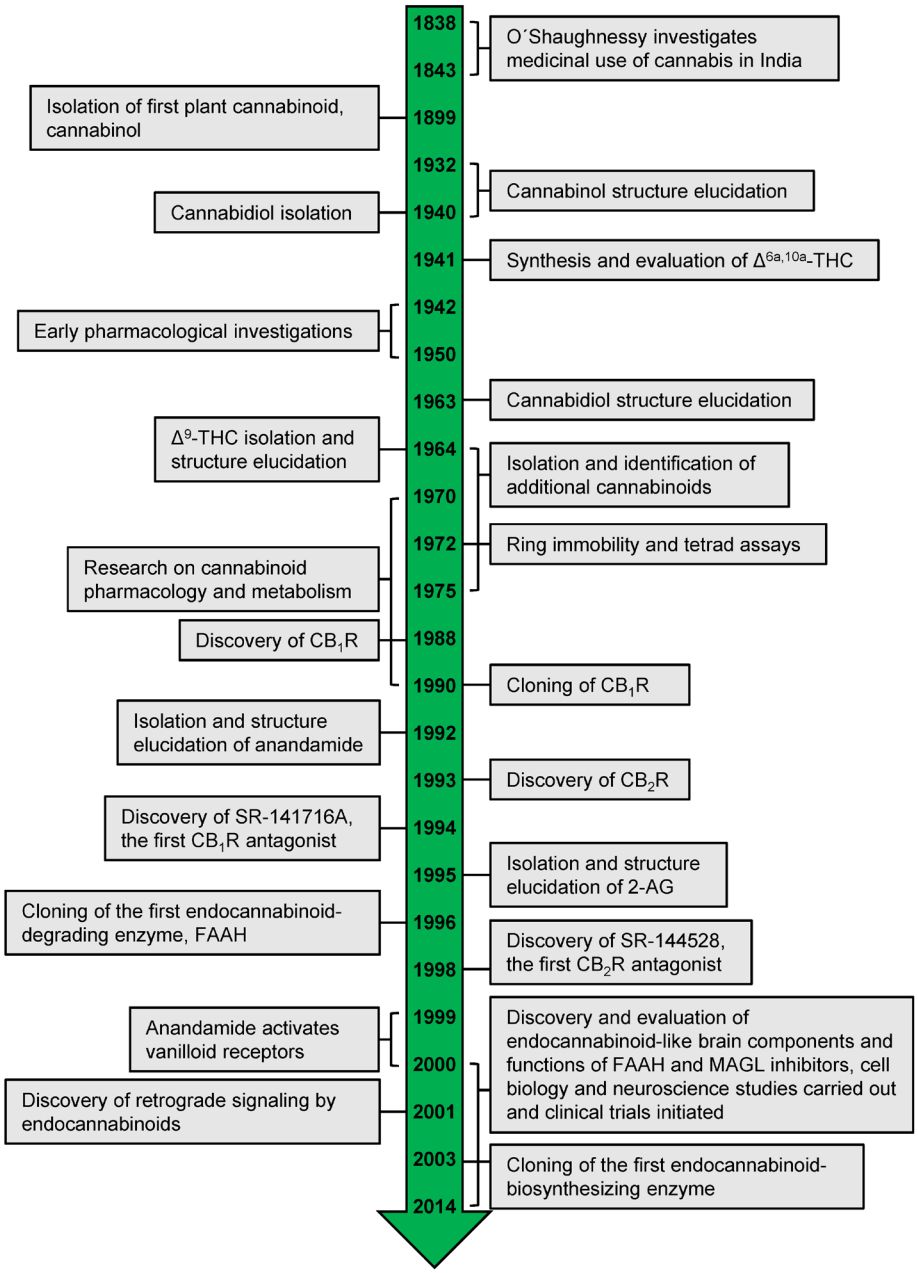


Figure 6. Timeline of cannabinoid and endocannabinoid research. From Mechoulam *et al.* 2014

These chemical advances promoted more research on the chemistry of the plant, the generation of a whole range of synthetic analogs structurally similar to THC and the isolation and identification of other phytocannabinoids (*i.e.* cannabinoids from the plant *C. sativa*), as well as a renewed interest in their neuropharmacology and biologic effects.

It was initially believed that due to its lipophilic nature, THC exerted its effects by a non-specific mechanism, perturbing cell membranes (Lawrence and Gill 1975). Its true mechanism of action was revealed more than twenty years after its identification by seminal works at Dr. Allyn Howlett´s laboratory. First, the discovery that cannabinoids were able to inhibit adenyl cyclase by acting through $G_{i/o}$ proteins, suggested the existence of a G protein coupled-receptor (GPCR) as a putative cannabinoid receptor (Howlett 1985). Second, the radioligand [^{3}H]-CP55940 (a synthetic cannabinoid produced by Pfizer) allowed the mapping by autoradiography of high-affinity cannabinoid binding sites in rat brain membranes (Devane *et al.* 1988). Confirmation of the existence of a cannabinoid selective GPCR came in 1990, with the cloning of the cannabinoid receptor type 1 (*Cnr1* or *CB1R*) on rat brain (Matsuda *et al.* 1990). Three years later, a second cannabinoid GPCR was cloned on spleen (Munro *et al.* 1993), and thus named cannabinoid receptor type 2 (*Cnr2* or *CB2R*).

It was obvious after these findings that cannabinoid receptors were not present in our body just to bind a plant constituent, but to be activated by specific endogenous ligands. In line with this idea, Dr. Mechoulam´s group isolated and identified a lipid on porcine brain, arachidonylethanolamide (AEA), that was able to bind CB1R with high affinity and mimic the behavioural actions of psychotropic cannabinoids when injected in mice (Devane *et al.* 1992). This was the first endocannabinoid to be identified and was named anandamide, based on the Sanskrit word ananda ("supreme joy"), and referring to its chemical structure (amide). A second endocannabinoid, 2-arachidonoylglycerol (2-AG), was subsequently isolated from peripheral tissues by Dr. Mechoulam´s group (Mechoulam *et al.* 1995) and Dr. Sugiura´s group (Sugiura *et al.* 1995).

Together, all these crucial discoveries led to the characterization of a new cell communication system, the so-called endocannabinoid system (ECS), which is involved in the control of a wide variety of biological functions such as motor behaviour, memory, pain, immunity, energy metabolism and reproduction, just to mention a few (Pacher, Bátkai, and Kunos 2006).

2.1. MAIN COMPONENTS OF THE ENDOCANNABINOID SYSTEM

The ECS consists of the cannabinoid receptors, their endogenous ligands (*i.e.* the endocannabinoids) and the proteins responsible of their synthesis, transport and degradation.

2.1.1. Cannabinoid receptors

To date, two canonical, well-characterized cannabinoid receptors (CBRs) have been described, namely CB1R and CB2R. The presence of these receptors is not-restricted to mammals, not even to vertebrates, which suggests that the ECS has been highly conserved during evolution and therefore

plays crucial roles in the physiology of living inviduals.

CB1R is encoded by the *CNR1* gene and consists of 472 amino acids in humans. Two recent studies have reported the crystal structure of the antagonist-bound CB1R (Hua *et al.* 2016; Shao *et al.* 2016), and the structural changes of this receptor upon agonist binding (Hua *et al.* 2017). CB2R is encoded by the *CNR2* gene, consists of 360 amino acid in humans, and its crystal structure has not been elucidated yet. These CBRs belong to the class A GPCR superfamily. They are integral membrane proteins with an extracellular N-terminal domain, followed by seven transmembrane domains and the C-terminal domain towards the cytoplasm. CBRs exhibit 42% amino acid sequence identity in humans, reaching almost 79% in the transmembrane residues (Munro *et al.* 1993). Both, CB1R and CB2R are typically coupled to heterotrimeric $G_{i/o}$ proteins, which suppress adenylyl cyclase and decrease cAMP levels (Howlett *et al.* 1986). Stimulation of CBRs also leads to the activation of ERK signaling pathway (*e.g.* by protein kinase A (PKA) inhibition (Davis *et al.* 2003), or by βɣ-dependent activation (Galve-Roperh *et al.* 2002). They can also signal in a G protein-independent manner through association with other molecules such as β-arrestin, a key mediator of GPCR desensitization and prolonged signaling. Nonetheless, CBR signaling is highly pleiotropic and deeply dependent on the cellular context and the nature and dose of the ligand. In fact, increasing evidence suggests that CBR signaling is far more intricate than it was initially believed. For example, biased signaling has been reported for CBRs, which means that different ligands acting on the same cannabinoid receptor in the same tissue, can give rise to markedly different cellular responses (Ibsen *et al.* 2017), and this is likely due to each ligand stabilizing different receptor conformations. Moreover, homo and heterodimerization not only between cannabinoid receptors but also with other GPCRs has also been described, and these interactions can greatly enrich the range of intracellular responses elicited by a ligand (Mackie 2005). In addition, as many others GPCRs, CBRs can also trigger growth factor receptor signaling cascades by transactivation of receptor tyrosine kinases (RTKs) (Dalton *et al.* 2009).

The main difference between CB1R and CB2R is their tissue and cell type distribution. CB1R is highly expressed in the central nervous system (CNS), especially in the cortex, olfactory bulb, hippocampus, basal ganglia, and cerebellum. In addition, it is highly expressed throughout the body, in locations as different as the cardiovascular system, the gastrointestinal tract, the liver or the muscles, among many others (Maccarrone *et al.* 2015). Thus, the once considered "central" cannabinoid receptor (due to its high expression levels in the CNS) is now recognized as an "ubiquitous" receptor. Unlike CB1R, CB2R is mainly expressed in cells and tissues of the immune system including the thymus, tonsils, B lymphocytes, T lymphocytes, macrophages, monocytes and natural killer cells, among others (Cabral and Griffin-Thomas 2009). CB2R expression has also been reported in other tissues such as retina (Lu *et al.* 2000) and vascular endothelium (Blazquez *et al.* 2003), and, more recently, within the brain, particularly in microglial cells and embryonic and adult neural progenitors (Palazuelos *et al.* 2006; Fernández-Ruiz *et al.* 2007). Of interest, increasing evidence demonstrates that the expression of both receptors is altered in certain pathological conditions, being cancer one of them (see below).

In addition to the two well-established GPCR cannabinoid receptors, several other receptors have been reported to be engaged and activated by certain cannabinoid compounds and related molecules. For example, the orphan receptor GPR55, the transient receptor potential channel 1 (*e.g.* vanilloid receptor or TRPV1), glycine receptors, nuclear receptors [*i.e.* peroxisome proliferator-activated receptors (PPARs)], ligand-gated ion channels (*e.g.* 5-HT3 receptors) and some voltage-gate Ca^{2+} and K^{+} channels (Pertwee 2015). However, none of them is strictly considered a canonical cannabinoid receptor according to the IUPHAR. The potential contribution of these receptors to the therapeutic effects of cannabinoids or the physiological effects of endocannabinoids are only beginning to be explored, and much less is known about how cannabinoid ligands regulate their signaling.

2.1.2. Endocannabinoids and their metabolism

Endocannabinoids (eCBs) are arachidonic acid-derived endogenous lipids that control basic biological processes such as neurotransmission, cell fate, immune response, energy homeostasis and reproduction, among many others. Anandamide is a partial agonist at CB1R and CB2R, showing less relative intrinsic affinity for CB2R. 2-AG shows greater potency and efficacy than AEA and is considered a CB1R and CB2R full agonist (Raphael Mechoulam *et al.* 2014).

It is widely accepted that eCBs are not stored in vesicles like other neurotransmitters but are rather synthesized "on demand" from complex lipids present at cell membranes when they are needed. Both compounds, AEA and 2AG, act on the same or neighboring cells as autocrine or paracrine mediators. AEA is synthesized from membrane phospholipid precursors (phosphatidylcholine and phosphatidylethanolamine) by the sequential action of an N-acyltransferase (NAT), which generates N-arachidonoylphosphatidylethanolamine (NAPE), and an N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Marzo *et al.* 1994) (Figure 7). On the other hand, 2-AG is generated from diacylglycerol (DAG) by a *sn*-1-selective DAG lipase. Two DAG lipase isozymes, α and β, have been cloned (Bisogno *et al.* 2003). Upon synthesis, eCBs are released *via* diffusion through the plasma membrane, possibly facilitated by an unidentified membrane carrier protein. For inactivation, they are transported back inside the cell and hydrolyzed by specific enzymes. AEA is hydrolyzed to arachidonic acid and ethanolamine mainly by a fatty acid amide hydrolase (FAAH) (Marzo *et al.* 1994), and 2-AG is hydrolyzed to arachidonic acid and glycerol mainly by a monoacylglicerol lipase (MAGL) (Dinh *et al.* 2002). Alternatively to those hydrolytic routes, AEA and 2-AG can be oxidized by cyclooxygenase-2 (COX-2), lipoxygenases (LOXs), or cytochrome P450 proteins (CYPs) (Figure 7).

2.1.3. Plant and synthetic cannabinoids

There are three classes of cannabinoids depending on their origin: phytocannabinoids (from *C. sativa*), endocannabinoids (produced by animals) and synthetic cannabinois (produced in the laboratory).

Among the more than 100 phytocannabinoids, the main psycoactive component is THC. It is agonist of both CBRs and it widely mimics the actions of endogenous cannabinoids. Other phytocannabinoids

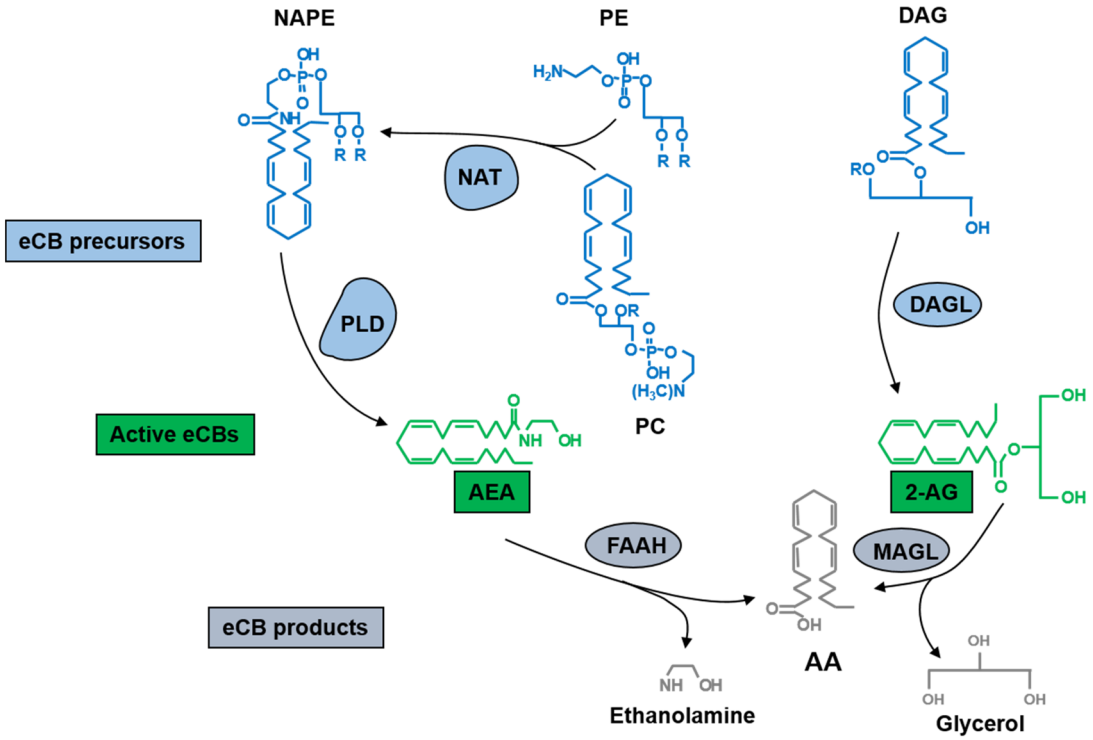


Figure 7. Synthesis and degradation of AEA and 2-AG. Main metabolic intermediates and enzymes involved in AEA and 2-AG synthesis and degradation. Adapted from Muccioli, 2010.

present in the plant are CBD, CBN, and cannabigerol (CBG), just to mention a few. CBD has very low affinity for the two CBRs, but it shows some cannabimimetic actions attributed to antioxidant properties, inhibition of anandamide degradation, and/or interactions with other cannabinoid receptors such as GPR55 (Gómez-Ruiz *et al.* 2007). CBD can also act as a CB_1R negative allosteric modulator (Laprairie *et al.* 2015)

The third group is formed by synthetic cannabinoids, invaluable pharmacological tools for the study of the ECS and with potential therapeutic applications. They are classified according to their chemical structure. Thus, we can distinguished: classical cannabinoids [structurally similar to phytocannabinoids, *e.g.* JWH-133, a CB_2R agonist (Huffman *et al.* 1999)]; non-classical cannabinoids [initially synthesized by Pfizer, *e.g.* CP-55,949 (Martin *et al.* 1991)]; aminoalkylindols [initially developed by Steling Winthrop, *e.g.* WIN55212-2, a CB_1R and CB_2R agonist (Pacheco *et al.* 1991)] and diacylpirazols [initially developed by Sanofi-Aventis, *e.g.* the CB_2R antagonist SR144528 (Rinaldi-Carmona *et al.* 1998), widely used in this Thesis].

2.2. THE ENDOCANNABINOID SYSTEM AND CANCER

During the last decades, numerous studies have described the important role of the ECS in multiple biological functions. It is therefore not surprising that an increasing body of evidence shows that this system is altered in many different diseases, including cancer (Velasco *et al.* 2012). It is not clear however, whether these alterations are the cause of some of those pathological status, as

suggested for migraine, fibromyalgia, irritable bowel disease and other syndromes (Russo 2016), or an adaptative attempt of the organism to recover homeostasis.

2.2.1. Role of cannabinoid receptors in cancer

Both CB1R and CB2R are generally upregulated in tumor tissues compared with their non-tumor counterparts, although there are some exceptions that may be tumor type-specific (Fraguas-Sánchez *et al.* 2018). There are also some studies showing alterations in endocannabinod levels and in the enzymes responsible for their degradation in several carcinomas (Velasco *et al.* 2012). However, the vast majority of research in this field has focused on evaluating the possible impact of an altered CBR overexpression in the outcome of cancer disease. There are two main hypotheses that try to explain the biological significance of these alterations: on the one hand, the upregulation of the ECS could be pathogenic, favoring tumor progression. On the other hand, an increased ECS activity could be the consequence of an attempt of the body to fight against disease, thus slowing down cancer progression. As expected, reports about the role of the ECS in cancer are conflicting, pointing in both directions -oncogenic or tumor suppressor- depending on the tumor type and context (Velasco *et al.* 2016) (see Table 1).

Supporting an oncogenic role of the ECS, the analysis of CB1R and CB2R mRNA expression from different human breast cancer samples revealed that the levels of the former were similar in cancerous tissue compared to non-tumor tissue, while CB2R expression was much higher than CB1R expression in all tumors, and almost undetectable in non-cancerous tissue. Moreover, CB2R mRNA levels seemed to increase in parallel with the histological grade of the tumors, suggesting a correlation with tumor aggressiveness. This last observation was further confirmed after analysing CBR protein expression in an additional and larger series of human breast cancer samples (Caffarel *et al.* 2010). The association between high CB2R expression and tumor aggressiveness has also been observed in other types of tumors, such as colorectal cancer (Martinez-Martinez *et al.* 2015), head and neck squamous cell carcinoma (Nulent *et al.* 2013) and renal cell carcinoma (Wang *et al.* 2017). In all these independent studies, high CB2R expression was associated with poor patient prognosis. In agreement with these observations, CB2R downregulation in renal cell carcinoma cells lines produced an impairment in cell proliferation and migration (Wang *et al.* 2017). In other types of tumors, such as glioblastoma, a combined up-regulation of CB1 and CB_2 receptors has been described; specifically, in high grade tumors compared with low grade tumors or healthy tissue (Wu *et al.* 2012). Similar results were found in prostate cancer (Sarfaraz *et al.* 2005; Orellana-Serradell *et al.* 2015), pancreatic cancer and ovarian carcinomas, where overexpression of both receptors was detected, and high CB1R expression was associated with disease severity and worse outcome (Chung *et al.* 2009; Messalli *et al.* 2014; Michalski *et al.* 2008). CB1R has also been proposed to be a bad prognosis factor in patients with stage II microsatellite-stable (Gustafsson *et al.* 2011) or following surgery in stage IV colorectal cancer (Jung *et al.* 2013). Co-expression of both cannabinoid receptors has also been detected in hepatocellular carcinoma, although their specific role in tumor progression is controversial. Some studies point to CB1R as a hepatocarcinogenesis driver

(Mukhopadhyay *et al.* 2015; Suk *et al.* 2016), whereas others suggest that both CBRs correlate with improved patient prognosis (Xu *et al.* 2006).

TUMOR TYPE	CB_1R	CB_2R	RELATION TO DISEASE OUTCOME	REFERENCES
Glioma	↑	↑	• CBR overexpression has been related to high tumor grade	(Sanchez *et al.* 2001; Wu *et al.* 2012)
Breast cancer	-	↑	• CB_2R is overexpressed in HER2+ tumors, and correlates with disease severity • CB_2R overexpression is a good prognosis marker in ER- and ER+ tumors	(Caffarel *et al.* 2006, 2010) (Elbaz *et al.* 2016)
Prostate cancer	↑	↑	• Overexpression of CB_1R is a negative marker of the disease outcome	(Sarfaraz *et al.* 2005; Chung *et al.* 2009; Orellana-Serradell *et al.* 2015)
Colorectal cancer	↑ or ↓	↑	• Genetic ablation of CB_1R increases intestinal tumor growth • High CB_1R expression is a bad prognosis marker following surgery in stage IV colorectal cancer • CB_2R upregulation is a poor prognosis factor	(Wang *et al.* 2008) (Jung *et al.* 2013) (Martinez-Martinez *et al.* 2015)
Pancreatic cancer	↑	↑	• High CB_1R levels are associated with bad prognosis	(Carracedo *et al.* 2006; Michalski *et al.* 2008)
Ovarian cancer	↑	↑	• CB_1R overexpression is associated with higher tumor aggressiveness	(Messalli *et al.* 2014)
Head and neck squamous cell carcinoma	-	↑	• High CB_2R expression is associated with reduced disease-specific survival	(Nulent *et al.* 2013)
Renal cell carcinoma	-	↑	• CB_2R overexpression is a marker of poor prognosis	(Wang *et al.* 2017)
Tongue squamous cell carcinoma	↑	↑	• CB_1R expression is a positive marker of disease outcome	(Theocharis *et al.* 2016)
Hepatocarcinoma	↑	↑	• CB_1R and CB_2R expression correlates with improved prognosis of patients • CR_1R is a hepatocarcinogenesis driver	(Xu *et al.* 2006) (Mukhopadhyay *et al.* 2015; Suk *et al.* 2016)

Table 1. Alterations of CB_1R and CB_2R in several carcinomas.
In the table: (-) denotes no difference or not studied, ↑ higher levels, ↓ lower levels of expression compared to normal tissue. Adapted from Fraguas-Sánchez *et al.* 2018

Increasing evidence suggests that the putative cannabinoid receptor GPR55 might play a role in cancer development. Thus, overexpression of GPR55 has been found in different types of cancer, and its implication as an inducer of tumorigenesis has been shown *in vitro* and/or *in vivo* models of glioblastoma (Andradas *et al.* 2011), prostate, ovarian (Piñeiro *et al.* 2011), skin carcinoma (Pérez-Gómez *et al.* 2013), breast (Andradas *et al.* 2016) and non-small lung cancer (He *et al.* 2015).

Altogether, these data support the hypothesis that the ECS is a pro-tumorigenic factor in cancer. Further strengthen this notion, the genetic deletion of CB1R and CB2R decreases UV light-induced skin carcinogenesis (Zheng *et al.* 2008), and CB2R overexpression enhances predisposition to leukaemia following leukaemia virus infection (Joosten *et al.* 2002).

On the other hand, different observations suggest that the ECS plays a tumor-suppressor role in different types of cancer. For example, CB2R overexpression in ER+ and ER- human breast cancer tumors was associated to a better patient prognosis (Elbaz *et al.* 2016). Other studies support the role of CB1R as a tumor suppressor, for example, in squamous tongue carcinoma, this receptor is a positive marker for disease-free survival (Theocharis *et al.* 2016), and its genetic inactivation increases intestinal tumor growth in a genetic mouse model of colon carcinoma (Wang *et al.* 2008).

In summary, all these studies demonstrate an abnormal expression of CBRs in oncologic contexts. However, further studies involving genetic or pharmacological manipulation of the ECS are required to clarify the specific tumoral context that tips the balance towards a suppressor role of the ECS or towards an inducer of tumorigenesis.

2.2.2. Antitumoral action of cannabinoids

Despite the conflicting data on the role of the ECS in tumor generation and progression, there is convincing evidence demonstrating that pharmacological activation of the ECS (either with CB1R or CB2R agonists or by endocannabinoid degradation inhibition) leads to anti-tumor responses. The first evidence showing anti-tumoral properties of cannabinoids was reported in 1975 by Munson *et al*, who found that THC inhibited lung adenocarcinoma cell growth *in vitro* and *in vivo* (Munson *et al.* 1975). Further research supporting this effect was not published until the late 1990s (Petrocellis *et al.* 1998; Sánchez *et al.* 1998). Since then, an avalanche of studies performed with several plant-derived (*e.g.* THC and CBD), synthetic (*e.g.* WIN-55,212-2 and HU-210) and endogenous cannabinoids (*e.g.* AEA and 2-AG) have shown anti-tumoral actions in a wide variety of preclinical models of different types of cancer, including breast, pancreas, lung or liver adenocarcinomas, glioblastomas or melanomas, among others [reviewed in (Velasco *et al.* 2012)]. The solidness of these studies set the bases for the first controlled clinical study of the safety and efficacy of the combination of a cannabis-based medicine with an anticancer drug in cancer patients. Specifically, a phase 2 placebo-controlled clinical trial was performed in hospitals of the United Kingdom and Germany, in patients with recurrent glioblastoma multiforme, to address some safety and efficacy endpoints of the combination of Sativex (*i.e.* a cannabis extract containing approximately equal amounts of THC and CBD) with temozolomide (*i.e.* an alkylating agent that constitutes the standard first-line treatment for this type of brain tumor). At the

time this Thesis was presented, the results of that study had not been published yet. However, a press release of the sponsor company has partially unveiled positive results (https://www.gwpharm.com).

Cannabinoids block tumor progression at different levels: 1) inhibition of cancer cell proliferation and induction of apoptosis, 2) impairment of tumor angiogenesis and 3) blockade of the metastatic process (Figure 8).

1) Effect on cancer cell proliferation / death

Cannabinoid-mediated inhibition of cell fate is due to their capacity to modulate several pathways involved in control of cell proliferation and apoptosis. For example, cannabinoids have been shown to induce cell cycle arrest. In breast cancer, CB2R agonists block cell cycle progression in the G2/M phase through downregulation of Cdc2 (Caffarel *et al.* 2006), and AEA induces S phase arrest through the inhibition of Cdk2 activity (Laezza *et al.* 2006). Similar results have been observed in other types of cancer, such as hepatocellular carcinoma (Xu *et al.* 2015), gastric (Park *et al.* 2011) or prostate cancer (Sarfaraz *et al.* 2005), after the activation of CBRs. Inhibition of Akt upon cannabinoid treatment has been reported as a main mechanism of inhibition of cancer cell proliferation in different types of cancer (Pisanti *et al.* 2013). Regarding the induction of cancer cell death, the first evidence of apoptosis induced by cannabinoids was reported in glioblastoma cells (Sánchez *et al.* 1998). Different studies have shown that, in these cells, THC and other cannabinoids bind to cannabinoid receptors, which leads to the accumulation of the pro-apoptotic sphingolipid ceramide (Galve-Roperh *et al.* 2000), and the subsequent activation of an ER stress-related signaling route that involves the up-regulation of the stress-regulator protein p8 and its effector, the pseudo- kinase tribbles homolog 3 (TRIB3) (Carracedo *et al.* 2006). The stimulation of this pathway, results in an inhibition of the Akt/mammalian target of rapamycin complex 1 (mTORC1) axis (Salazar *et al.* 2009), that leads to autophagy and mitochondria-driven apoptosis. This seems to be a quite general mechanism of cancer cell death induced by cannabinoids since it has also been reported in other types of cancer such us pancreatic cancer (Carracedo *et al.* 2006), melanoma (Armstrong *et al.* 2015) or hepatocarcinoma (Vara *et al.* 2011), among others. Additional cell signaling pathways involved in cannabinoid-induced anti-proliferative and pro-apoptotic effects include the activation of different kinases, such as p38 MAPK, c-Jun N-terminal kinase (JNK), and ERK1/2 (Herrera *et al.* 2005; Rueda *et al.* 2000). Moreover, cannabinoid receptor-independent mechanisms have been described for the antitumoral action of cannabinoids. Notably, CBD and other marijuana-derived cannabinoids have been proposed to promote the apoptotic death of cancer cells acting independently of CB1R and CB2R, through the generation of reactive oxygen species (ROS) in cancer cells (Massi *et al.* 2006; Singer *et al.* 2015). It has also been proposed that CBD may activate TRPV2 receptors to promote cancer cell death (Nabissi *et al.* 2012).

It is important to note that the induction of cell death is selectively exerted on cancer cells. Thus, the viability of non-cancerous cells from different origins in culture is not affected by cannabinoid treatment (Velasco *et al.* 2012). Moreover, these compounds can produce cytoprotective effects

against diverse cytotoxic insults in other physiological contexts. Thus, cannabinoids have been shown to protect neurons from toxic insults such as excitotoxicity, ischemia, neurodegenerative diseases, etc. both *in vitro* and *in vivo* (Fernández-Ruiz *et al.* 2015). In addition, and supporting their lack of toxicity, multiple clinical trials with cannabis-based medicines have been performed during the last years in thousands of cancer patients for the management of pain, nausea and vomiting, and they all have proved the safety of these compounds.

2) Impairment of tumor angiogenesis

A critical step in cancer progression is the generation of a new vascularization network (neoangiogenesis) to provide nutrients, gas exchange and waste disposal to the growing tumor. Therefore, targeting neoangiogenesis constitutes one of the most promising therapeutic approaches against cancer. The first study addressing the impact of cannabinoids on tumor vascularization reported that tumors treated with the CB2R-selective agonist JWH-133 showed a pattern of blood vessels characterized by very small, narrow and impermeable capillaries when compared to vehicle-treated tumors, in murine models of glioblastoma (Blázquez *et al.* 2003). These results were corroborated soon in other types of tumors, such as skin cancer (Casanova *et al.* 2003), and thyroid carcinomas (Portella *et al.* 2003).

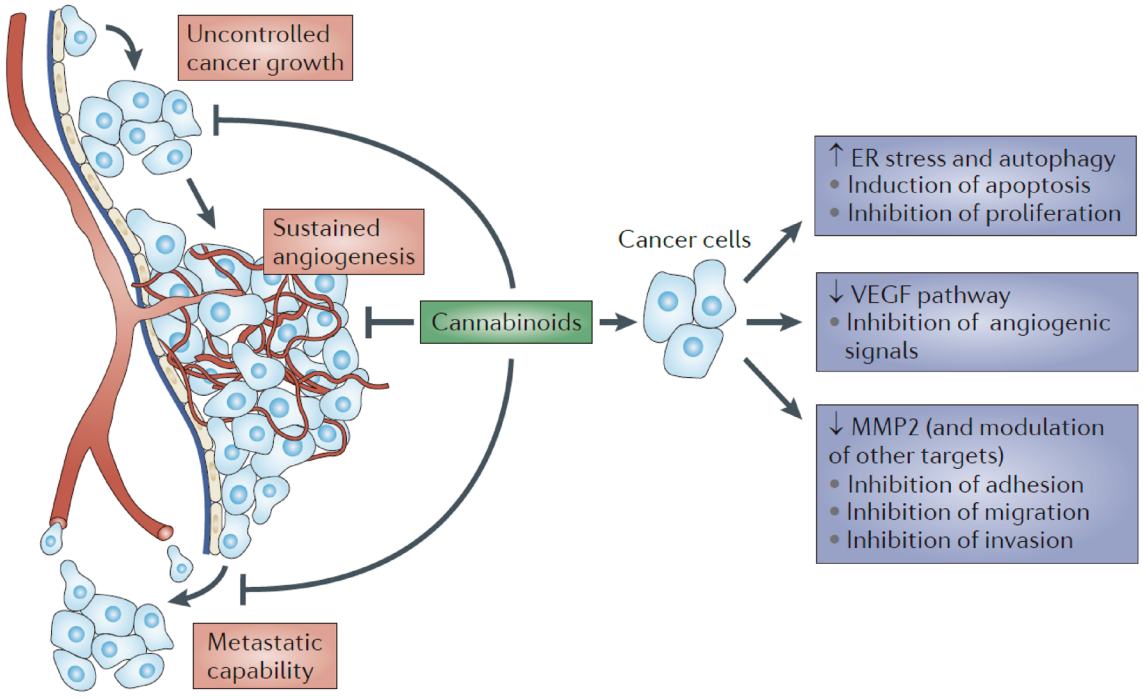


Figure 8. General mechanisms of cannabinoids anti-tumor action. Cannabinoids induce antitumoral responses by three main mechanisms: 1) inhibition of cancer cell proliferation and induction of cancer cell apoptosis, 2) impairment of angiogenesis and 3) inhibition of metastasis. From Velasco, Sánchez, and Guzmán 2012.

Two main mechanisms of action have been proposed to explain the anti-angiogenic properties of cannabinoids. On the one hand, the activation of cannabinoid receptors on vascular endothelial cells

inhibits their migration and survival, thus preventing blood vessel formation. On the other hand, activation of cannabinoid receptors on tumor cells blocks the production of proangiogenic proteins such as angiopoietin-2, placental growth factor and vascular endothelial growth factor (VEGF) (Blazquez *et al.* 2003). More recently, Ramer and colleagues have demonstrated that cannabinoids also inhibit angiogenic capabilities of endothelial cells *via* release of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) from lung cancer cells (Ramer *et al.* 2014).

3) Inhibition of metastasis

During neoplastic progression, some cancer cells acquire the ability of escaping the primary tumor mass, and are able to migrate and invade distant tissues, generating metastasis, the main cause of cancer-associated deaths. It has been described that cannabinoids, by activating cannabinoid receptors, inhibit adhesion, migration and invasiveness of different cancer cell lines in culture [reviewed in (Velasco *et al.* 2016)]. In addition, these compounds reduce the formation of distant tumor masses in animal models of cancer. Thus, cannabinoid intraperitoneal administration reduced the number of metastasic nodes in xenograft-based models of melanoma (Blázquez *et al.* 2006), lung cancer (Portella *et al.* 2003) and breast cancer (Grimaldi *et al.* 2006). This effect has also been observed in the MMTV-neu mouse, a clinically relevant model of HER2-driven metastatic breast cancer. These animals develop breast tumors and metastasis in the lungs. Treatment with the CB2R-selective agonist JWH-133 significantly reduced not only tumor growth but also the percentage of animals that generated lung metastasis (Caffarel *et al.* 2010). The antimetastatic effects of cannabinoids relies, at least in part, on the modulation of extracellular proteases, such as matrix metalloproteinase 2 (MMP2), and their inhibitors, such as TIMP1, *via* activation of either CB1R or CB2R, depending on the tumor type (Blazquez *et al.* 2008; Ramer and Hinz 2008). For CBD however, the best described mechanism of antimetastatic action involves downregulation of the transcription factor ID-1 (inhibitor of DNA binding-1), and effect that is independent of cannabinoid receptor activation (McAllister *et al.* 2007; Soroceanu *et al.* 2013).

2.2.3. Protumoral action of cannabinoids

Although the vast majority of reports published so far shows that cannabinoids induce antitumor responses in many types of cancer models, a few articles have reported protumor actions in response to cannabinoids. Thus, McKallip and coworkers observed that THC can lead to enhanced tumor growth when cancer cells express low to undetectable levels of cannabinoid receptors, by specifically suppressing the antitumor immune response (McKallip *et al.* 2005). Similar results were obtained in lung cancer, where THC treatment accelerated tumor growth in immunocompetent mice but not in SCID mice (Zhu *et al.* 2000). On the contrary, a study in melanoma showed that treatment with cannabinoids inhibited tumor growth both in immune-competent and immune-deficient (nude) mice (Blázquez *et al.* 2006). Additional research is warranteed to clarify the contribution of the immune system to cannabinoid antitumoral action.

A pro-proliferative effect of cannabinoids in culture cells has also been reported. Takeda and co-workers, for example, found that THC enhances the proliferation of MCF7 cells trough a cannabinoid-receptor independent mechanism due to the lack of detectable levels of CB1R and CB2R (Takeda *et al.* 2008). This finding is in line with that reported by McKallip *et al.* (see above), and suggests that the absence of cannabinoid receptors may explain the protumoral effect of cannabinoids observed by these authors. Additional studies are needed to determine whether these observations may have any impact on actual patients, in whome an overexpression of cannabinoid receptors is widely observed (Fraguas-Sánchez *et al.* 2018).

Of interest, other groups have reported a biphasic effect of cannabinoids on the viability of different cancer cells. Thus, low cannabinoid concentrations -in the nM range- have been shown to promote cell proliferation, whereas higher concentrations, -in the μM range- induce antiproliferative responses in the same cancer cells (Sanchez *et al.* 2003; Hart *et al.* 2004; Martínez-Martínez *et al.* 2016;). In these studies, all cell lines used expressed cannabinoid receptors, but the mechanism underlying this biphasic effect is still unclear. Of interest, the biphasic effect of these compounds has been observed in many different scenarios (Sulcova, 1985) such as the control of appetite (Bellocchio *et al.* 2010) or on anxiety (Rey *et al.* 2012) and it has been hypothesized that could be due to allosteric modulation of the CBRs or to differential coupling to G proteins at low or high concentrations of cannabinoids.

3. MEMBRANE RECEPTOR HETEROMERS

Signal transduction is a research field in permanent evolution, and classically assumed paradigms are constantly being revisited. For example, the classical model of one ligand, one receptor and one set of signal transduction events in a linear manner is being replaced by models involving higher order complexes. In fact, it is currently clear that signal integration and diversification arises from complex networks involving cross-communication between different signaling units. GPCRs and RTKs are an excellent example. These are the main receptors at the plasma membrane and are in charge of transducing extracellular stimuli into intracellular signaling cascades that control key cell functions. It is increasingly evident that they can signal as parts of macromolecular complexes and not only as separate units. Most RTKs, as previously mentioned, need to dimerize to trigger intracellular signaling cascades. GPCRs, on the other hand, do not usually require dimerization to be active, but it has been described that they form homo and heterodimers with other GPCRs. Moreover, a functional crosstalk between GPCRs and RTKs has been extensively reported, and recent work suggests a physical interaction between them with potential consequences on cell signaling. Cannabinoid receptors, as it will be discussed in detail below, are not an exception, and they have been shown to form homo and heterodimers with each other and with other GPCRs (Mackie 2005, Callén *et al.* 2012), as well as transactivate RTKs, suggesting at least indirectly, that they may also interact phyisically (Dalton and Howlett 2012).

The classically accepted definition of a receptor heteromer is a “macromolecular complex composed of

at least two functional receptor units (protomers) with biochemical properties that are demonstrably different from those of its individual components" (Ferré *et al.* 2009). The discovery and signaling characterization of these receptor complexes have changed our understanding on the biochemistry and pharmacology of GPCRs, and might have a big impact on drug discovery. With an increase in the number of reports identifying GPCR heteromers, distinguishing receptors localized in the same cell undergoing functional cross-talk from receptors forming physiologically relevant heteromers has become an important challenge. Thus, three consensus criteria have been proposed to congently define true heteromers in endogenous systems (Ferré *et al.* 2009; Kenakin *et al.* 2010; Gomes *et al.* 2016) (Figure 9) :

1) Physical receptor-receptor interactions in native tissue. Classically, most dimerization studies have used immunoprecipitation, fluorescence complementation and resonance energy transfer approaches (Ferré *et al.* 2009). Nonetheless, the majority of these techniques rely on artificial systems and are technically difficult to perform in native tissue. To demonstrate the interaction between receptors in native tissue, colocalization and coimmunoprecipitation studies have been frequently used (Gomes *et al.* 2016). However, colocalization does not provide enough subcellular resolution to establish close proximity, and even coimmunoprecipitation can occur with receptors located too far away to directly modulate one another. More recently, proximity ligation assay (PLA) has become the technique of choice for demonstrating the proximity of a variety of proteins, including GPCRs, in native tissue. This is a technique based on antibody detection that allows the visualization of the interacting proteins by fluorescence microscopy if they are located less that 17 nm one from each other (see Materials & Methods for further details) (Fredriksson *et al.* 2002).

2) Specific heteromer´s biochemical fingerprint. This includes distinctive pharmacological, signaling and trafficking properties (Milligan *et al.* 2006). Thus, some studies have reported that GPCR heteromerization can (1) alter the binding properties of protomer-selective ligands (Kabli *et al.* 2010); (2) change signal transduction of the individual protomers such as G-protein activity (Fan *et al.* 2005), AC activity, ERK phosphorylation or β-arrestin signaling (Rozenfeld and Devi 2007); (3) modulate the trafficking properties of the protomers such as subcellular localization or agonist-induced internalization (Hasbi *et al.* 2007).

3) Physiological or pathological relevance of the heteromers *in vivo*. For this purpose, tools aimed at disrupting or modulating the heteromer should be used. For example:

- Heteromer selective antibodies: immunoglobulins that selectively recognize an epitope in the heteromer but not in the individual protomers. This tool is useful to detect the expression levels of a heteromer under different physiological or pathological conditions. Such antibodies can also block heteromer-mediated signaling and, therefore, could conceivably have therapeutic potential (Gomes *et al.* 2014).

- Membrane-permeable peptides: the aim of these tools is disrupting the physical interaction between the proteins that form the heteromer. This can be achieved by using the so-called TM peptides: a small

peptide with sequence homology to the heteromer interface, fused to another peptide that makes the construction membrane-permeable (Viñals *et al.* 2015).

- Mice expressing dimerization-deficient mutants: transgenic animals expressing receptor protomers that are unable to dimerize have also helped to support the involvement of heteromers in specific functions *in vivo* (González *et al.* 2012)

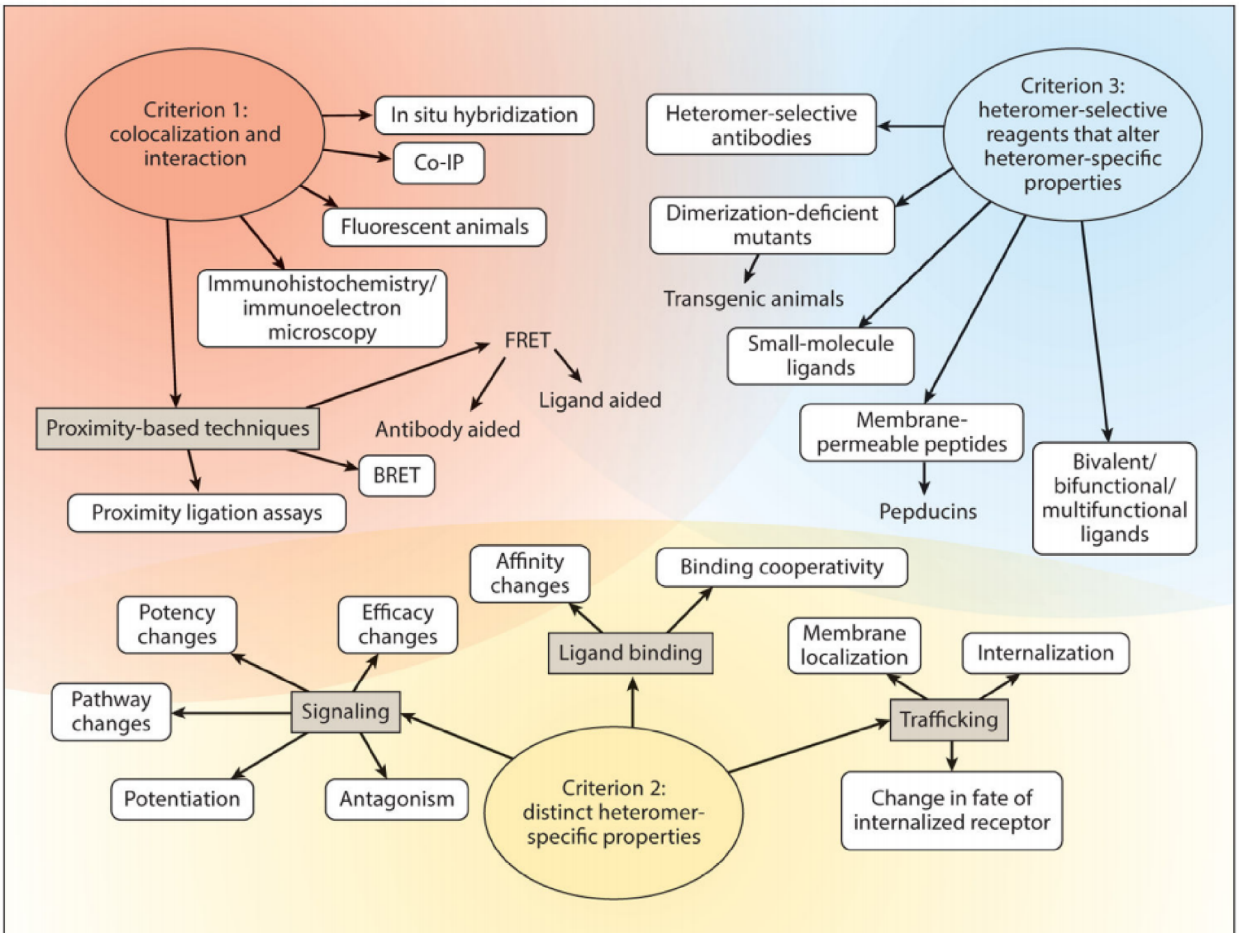


Figure 9. The three criteria to establish GPCR heteromerization and approaches to address them. Criterion 1 requires evidence of receptor-receptor colocalization and interaction in native tissues. Criterion 2 requires de demonstration of distinct and specific biochemical properties of the heteromer. Criterion 3 requires the disruption of the heteromer by selective reagents to alter the heteromer-specific properties described in criterion 2. From Gomes *et al.* 2016

3.1. GPCR-GPCR HETEROMERS

GPCRs constitute the largest and possibly the most diverse superfamily of proteins represented in any eukaryotic cell. Historically, it was assumed that they function as monomeric entities. However, a growing body of evidence indicate that they can form homomers, heteromers and even higher oligomeric complexes (Milligan *et al.* 2006 ; Ferré *et al.* 2009). The first evidence of GPCR dimerization came in 1975 when Robert Lefkowitz demonstrated the negative co-operativity of β-adrenergic receptors, which was explained by the existence of β-adrenergic receptor homodimers (Limbird *et al.* 1975). Since then, an increasing number of studies have reported a physical interaction between

them, that generates unique signaling platforms with physio-pathological implications different than those of the forming monomers (Fuxe *et al.* 2007).

Most GPCR-GPCR heteromers have been described in the CNS. (Brugarolas *et al.* 2014; Guidolin *et al.* 2015) . Among the best characterized, and just to mention a few, heteromers have been found between opioid receptors DOR and MOR, which constitute a potential target to treat pain and related disorders (Fujita *et al.* 2015); dopamine D1 and D2 receptors in striatal neurons, which have been implicated in major depression (Pei *et al.* 2010); and adenosine A2A and dopamine D2 receptors, implicated in Parkinson disease, schizophrenia and drug addiction (Fuxe *et al.* 2010). GPCR heteromers have also been found in peripheral tissues, for example between cytokine and adrenergic receptors in vascular smooth muscle, with implications in blood pressure regulation (Tripathi *et al.* 2015), or between different GPCRs in distinct endocrine systems (Jonas and Hanyaloglu 2017), which may constitute new targets for endocrine-related issues.

Cannabinoid receptor-containing heteromers

CB1R and CB2R have been recently described as constituents of several GPCR heteromers. They can interact between them (Callén *et al.* 2012) and with other GPCRs. Due to the high abundance and importance of CB1R in the CNS, most of its heteromers have been described in that particular location. The CB1R-containing heteromers described so far include the following: (1) CB1R- serotonin $5HT_{2A}R$ heteromer, expressed in specific brain regions involved in the control of memory (Viñals *et al.* 2015); (2) CB1R- orexin OX1R heteromer, which may regulate appetite and feeding (Ward *et al.* 2011); (3) CB1R-adenosine $A_{2A}R$ heteromer, found in the dorsal striatum and implicated in motor behaviour (Ferré *et al.* 2010; Moreno *et al.* 2018); (4) CB1R-opioid MOR heteromer, which has emerged as a new candidate for antinociceptive therapy (Bushlin *et al.* 2010; Bushlin *et al.* 2012); (5) CB1R- orphan GPR55 heteromer, expressed in striatum, although its biological function has not been elucidated yet (Martínez-Pinilla *et al.* 2014), and (6) CB1R- angiotensin AT1R heteromer, as an example of a peripheral CB1R heteromer (Rozenfeld *et al.* 2011).

CB2R, on the other hand, has been shown to form heteromers with GPR55 (Balenga *et al.* 2014; Moreno *et al.* 2014) and with the chemokine receptor CXCR4 (Coke *et al.* 2016; Scarlett *et al.* 2018) in cancer cells. Specifically, we have previously described that CB2R-GPR55 heteromers are implicated in THC anti-tumoral action (Moreno *et al.* 2014). For CB2R-CXCR4, authors proposed that simultaneous agonist activation promotes the physical interaction of CXCR4 and CB2R, leading to a non-functional heterodimer on the plasma membrane of cancer cells, and an overall attenuation of tumor progression. In particular, they suggested that agonist-induced heterodimerization abrogates CXCR4 pro-tumoral actions by reducing the $G_{\alpha 13}$-RhoA signaling pathway, which results in cell migration and cell invasion inhibition (Scarlett *et al.* 2018). More recently, CB2R has been reported to form heteromers with the orphan receptor GRP18 in microglia cells, which may play a role in controlling neuroinflamation (Reyes-Resina *et al.* 2018).

3.2. GPCR-RTK FUNCTIONAL CROSSTALK

It is well established that the growth-promoting activity of many GPCRs involves at least in part the activation of RTKs and their downstream signaling cascades. These functional crosstalk between these two receptor superfamilies was first described in Rat-1 fibroblasts stimulated with an array of GPCR agonists, which induced a rapid tyrosine phosphorylation of EGFR (Daub *et al.* 1996). Such observations led to the emergence of the "transactivation" concept, which designates a phenomenon by which a given receptor is indirectly activated by a ligand of a heterologous receptor (Cattaneo *et al.* 2014).

GPCR-mediated RTK transactivation

This functional interaction between GPCRs and RTKs can occur by different molecular mechanisms (Figure 10):

- Ligand-dependent RTK transactivation: GPCR stimulation induces the activation of transmembrane matrix metalloproteinases (ADAMs) that produce the proteolytic cleavage of RTK pro-ligands that are bound to the cell membrane. This process generates a RTK ligand that binds and activates the corresponding RTK, thus leading to the activation of specific RTK-driven signaling cascades (Prenzel *et al.* 1999) (Figure 10A).
- Ligand-independent RTK transactivation: this alternative mechanism requires either the association of the GPCR and the RTK within a protein complex, and, or the phosphorylation of the RTK by a tyrosine kinase downstream of the GPCR (Figure 10B). For example, the SRC family of non-receptor tyrosine kinases is activated by GPCR agonists through direct interaction with specific cytoplasmic receptor domains, or by binding to GPCR-associated proteins, such as heterotrimeric G-protein βγ subunits or β-arrestins (Luttrell and Luttrell 2004). Then, these kinases can directly phosphorylate and activate RTKs (Biscardi *et al.* 1999).
- Another pathway that can contribute to RTK transactivation by GPCR ligands is the generation of reactive oxygen species (ROS), which in turn inactivate protein-tyrosine phosphatases, thereby increasing RTK activity (Fischer *et al.* 2004).

RTK-mediated GPCR transactivation

The molecular mechanisms described for RTK-mediated GPCR transactivation are similar to those employed by GPCR agonists to transactivate RTKs:

– In some cases, activation of RTKs leads to the generation and secretion of GPCR ligands. For example, activation of some RTKs, such as TrkA, triggers the synthesis of sphingosine-1-phosphate (S1P), which in turn activates S1P1 receptors (Toman *et al.* 2004). This mechanism may have important implications in breast cancer, as several reports demonstrate that a sphingosine kinase 1, the enzyme that syntheses S1P, regulates survival, proliferation and migration of breast cancer cells (Pyne *et al.* 2007).

– In other cases, the transactivation of GPCRs by RTKs occurs in a ligand-independent manner, and requires the physical interaction with intracellular scaffolding proteins, as well as the phosphorylation of the transactivated GPCRs (Figure 10C). As an example, after insulin stimulation, the IGF1R is able to directly phosphorylate the β2-adrenergic receptor (Baltensperger *et al.* 1996). It also induces the activation of c-SRC and its downstream Akt pathway, which in turn phosphorylates the β2-adrenergic receptor (Doronin *et al.* 2002). In the context of cancer , for example, IGF-1 transactivates the CXCR4 chemokine receptor in breast metastatic human cell lines, and this promotes chemotaxis and cell survival in the absence of the endogenous ligand for CXCR4 (Akekawatchai *et al.* 2005).

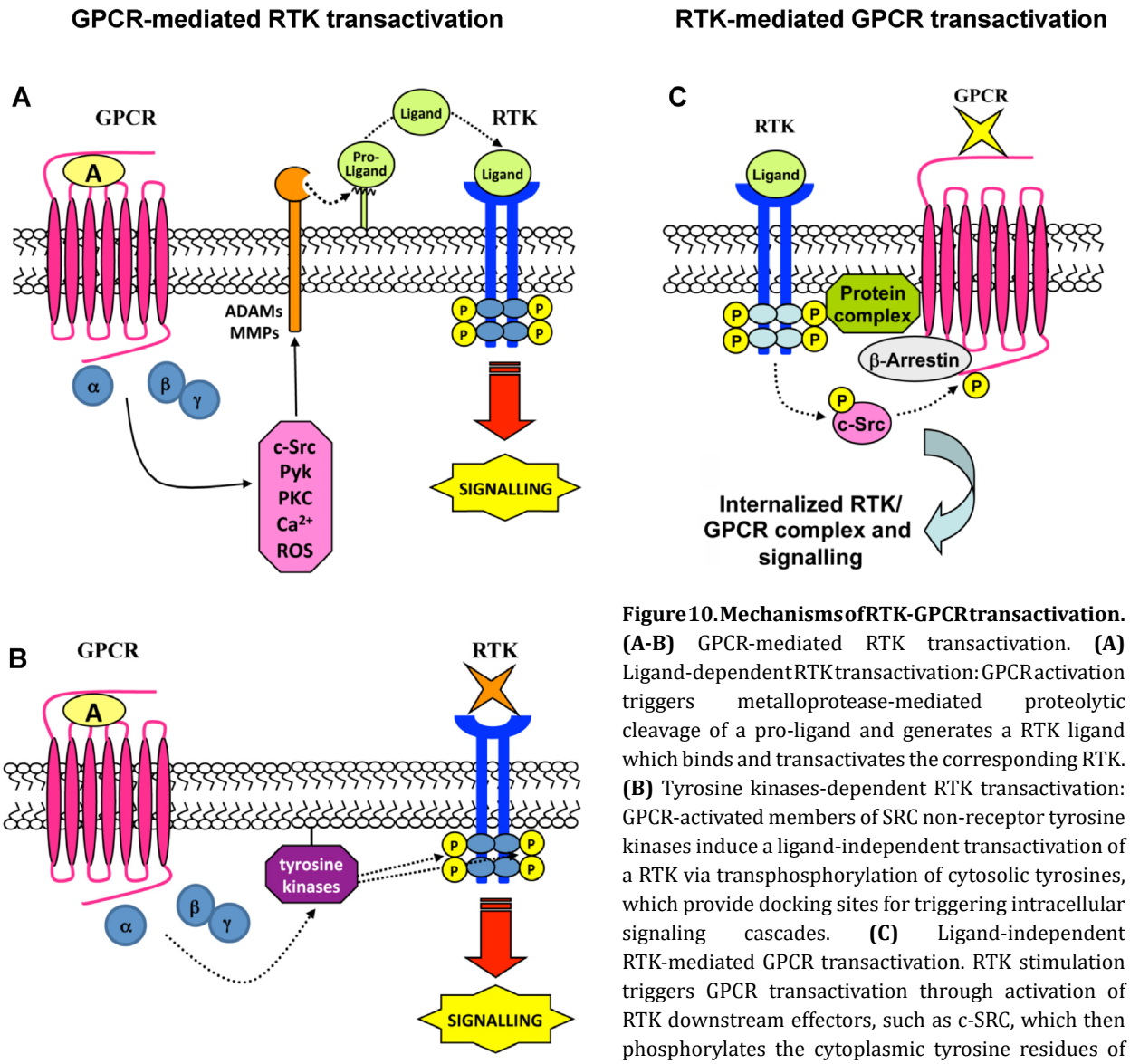


Figure 10. Mechanisms of RTK-GPCR transactivation. **(A-B)** GPCR-mediated RTK transactivation. **(A)** Ligand-dependent RTK transactivation: GPCR activation triggers metalloprotease-mediated proteolytic cleavage of a pro-ligand and generates a RTK ligand which binds and transactivates the corresponding RTK. **(B)** Tyrosine kinases-dependent RTK transactivation: GPCR-activated members of SRC non-receptor tyrosine kinases induce a ligand-independent transactivation of a RTK via transphosphorylation of cytosolic tyrosines, which provide docking sites for triggering intracellular signaling cascades. **(C)** Ligand-independent RTK-mediated GPCR transactivation. RTK stimulation triggers GPCR transactivation through activation of RTK downstream effectors, such as c-SRC, which then phosphorylates the cytoplasmic tyrosine residues of the GPCRs. This triggers β-arrestin recruitment, which in turn, promotes the internalization of the RTK/GPCR complex. From Cattaneo *et al.* 2014.

Cannabinoid receptors and RTK crosstalk

Crosstalk between CBRs and RTKs was first described in CHO cells. IGF1R activation by their endogenous ligands produced ERK activation, which was antagonized by a CB1R-selective antagonist (Bouaboula *et al.* 1997). Since then, several functional connection between CBRs and RTKs have been described, mostly with CB1R in the CNS. For example, CB1R activation by endocannabinoids regulates interneuron migration by transactivating the TKRB receptor trough a mechanism that involves SRC kinases (Berghuis *et al.* 2005).

In the context of cannabinoid receptors and cancer, the main topic of this Thesis, a functional crosstalk between CBRs and RTKs has also been reported (Dalton *et al.* 2009). In particular, activation of CBRs by different agonist (*e.g.* THC, AEA, JWH-015) inhibits EGF-induced growth and invasion of lung (Preet *et al.* 2008; Ravi *et al.* 2016), breast (Elbaz *et al.* 2016) and prostate cancer cells (Mimeault *et al.* 2003). On the contrary, Hart *et al.* reported that CB1R activation induces cell proliferation of glioblastoma and lung cancer cells by ERK activation, which was completely dependent on EGFR transactivation. In addition, the authors found that CB1R-induced EGFR transactivation was ligand-mediated *via* TACE/ADAM17 activation (Hart *et al.* 2004). Altogether, these findings provide evidence for a CBR-RTK functional crosstalk, with a potential relevance in the development and progression of cancer. Although some authors have suggested it, to the best of our knowledge no direct physical interaction between CBRs and RTKs has been reported yet.

3.3. GPCR-RTK HETEROMERS

It is therefore clear that a functional interaction between GPCRs and RTKs can occur, and this is manifested as transactivation responses that connect and diversify signal transduction pathways (Pyne and Pyne 2011). During the last few years the question has arised on whether this functional interaction involves the physical interaction between receptors. It has been proposed that some GPCRs associate with RTKs forming large receptor complexes, known as GPCR-RTK heteroreceptors (Borroto-Escuela *et al.* 2012). The physical interaction between both superfamilies of receptors could explain the aforementioned transactivation concept, but it also reveals the existence of novel functional signaling structures with biochemical and pharmacological properties distinct from those of the corresponding protomers. Although there are multiple examples of RTK-induced transactivation of GPCRs and *vice versa*, there are still few examples of possible RTK-GPCR heteromers. One of the first interactions reported was that formed by HER2 and the β2-adrenergic receptor in the heart (Negro *et al.* 2006). The authors demonstrated a physical interaction between them, and the requirement of HER2 for GPCR agonist-induced ERK activation in myocytes. Moreover, and although they did not provide any proof for that, the authors suggested that the interaction of HER2 or other members of the HER family with other GPCRs might regulate other important processes such as cancer progression. A partial support to this hypothesis came a few years later, when the GPR54-EGFR heteromer was described by FRET in cancer cells (Zajac *et al.* 2011). Specifically, a GPR54 ligand induced breast

cancer cell invasion and metalloproteinase (MMP)-9 production by transactivation of EGFR *via* β-arrestin II. Evidence for direct GPCR-RTK interaction has also been provided in other contexts, such in the CNS. Thus, heteromers formed by fibroblast growth factor receptor (FGFR) and adenosine A_{2A} receptor (Flajolet *et al.* 2008) or serotonin 5-HT1A receptor (Borroto-Escuela *et al.* 2015) have been described, and suggested to play a role in synaptic plasticity.

These complex molecular organization implies new signaling interconnected pathways controlling many physio/pathological responses, and provide additional options for therapeutic intervention. Therefore, GPCR-RTK heteromers may lead to novel therapeutic strategies such as specific blockade of RTK signaling by inhibiting the corresponding GPCR, *vice versa*, or targeting the heteromer *per se* (Borroto-Escuela *et al.* 2018; Pyne and Pyne 2011). It is tempting to speculate that this latter approach would produce less side effects than a direct RTK inhibition.

AIMS

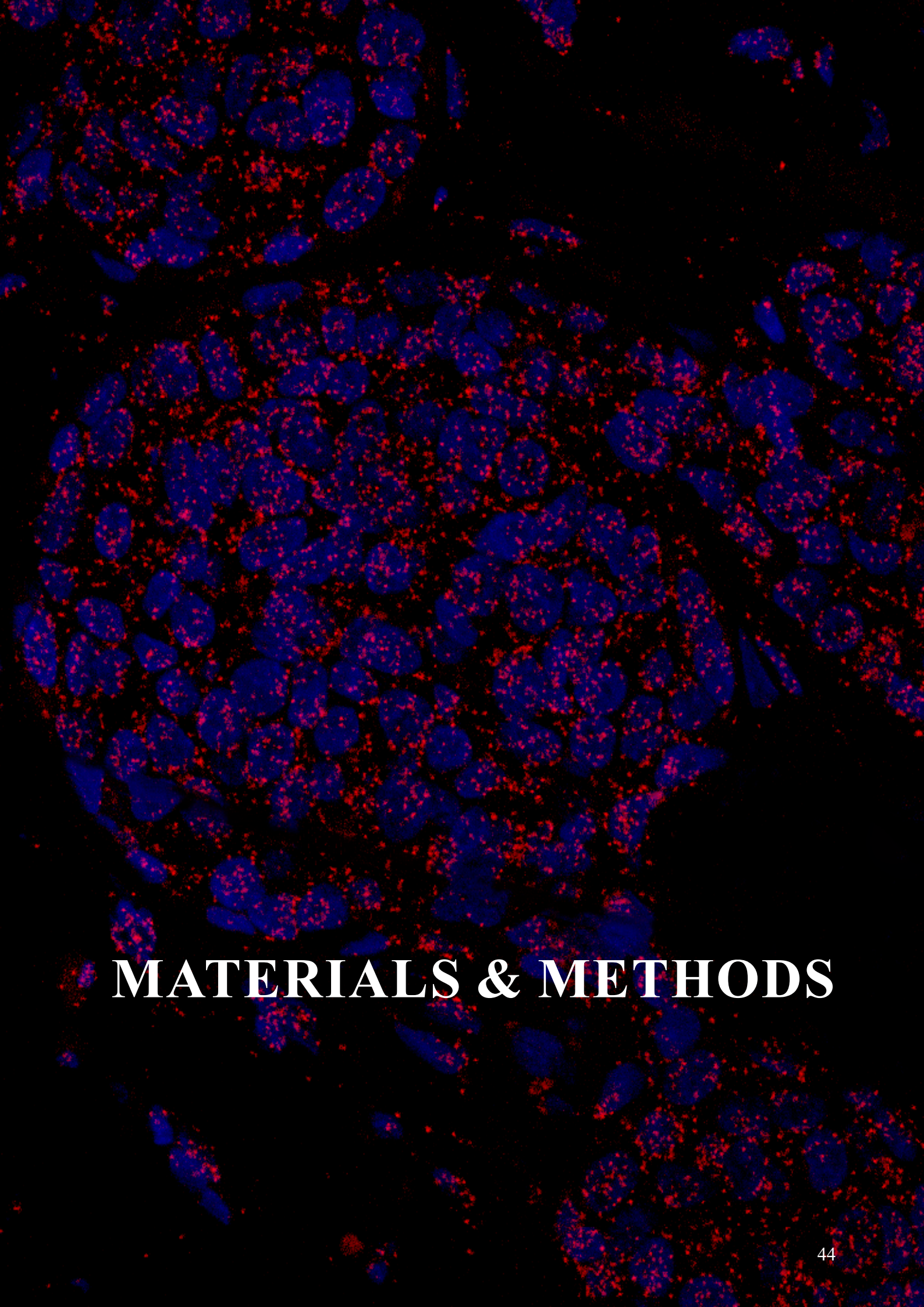
The endocannabinoid system is emerging as a promising target in anti-cancer therapy. Accumulating evidence has shown that its pharmacological activation (either with plant-derived or synthetic cannabinoids or by increasing the endocannabinoid tone) promotes antitumor responses in different preclinical models of cancer. In addition, alterations in the levels of endocannabinoids and/or their receptors have been reported in different types of human tumors when compared to the corresponding non-malignant tissue. However, little is known about the role of the ECS in tumor physio-pathology and the potential impact of these alterations on cancer development.

Previous results from our laboratory showed that CB2R expression is increased in human breast cancer compared to non-transformed mammary tissue, and that this overexpression is highly associated to HER2+ tumors. In this context, we defined the following **Aims** for this Doctoral Thesis:

Aim 1. Analysis of the role of CB2R in breast cancer generation and progression.

Aim 2. Analysis of the potential physical interaction between HER2 and CB2R in HER2+ breast tumors.

Aim 3. Analysis of HER2-CB2R heteromers as potential therapeutic targets in HER2+ breast cancer.



MATERIALS & METHODS

1. Reagents

THC was from THC Pharm GmbH (Frankfurt, Germany). For cell culture incubations, it was disolved in DMSO and, unless otherwise indicated, the THC concentration used in the assays was 3μM for HCC1954 cells and 4μM for BT474 and HEK293 cells. The c-SRC- selective inhibitor saracatinib (AZD0530) (Santa Cruz Biotecnology, Santa Cruz, CA), the CB2R-selective antagonist SR144528 (SR2) (Tocris Bioscience, Abingdon, UK), the MAPK kinase inhibitor UO126 (Calbiochem, San Diego, CA) and the proteasome inhibitor lactacystin (Calbiochem) were dissolved in DMSO, and added to the cell cultures (1μM) 1h prior THC.

2. Cell cultures

The following cell lines were from American Type Culture Collection (ATCC, Barcelona, Spain): MDA-MB-231, MDA-MB-468, MCF-7, T47D, BT474, MDA-MB-361, SKBR3, HCC1954 and AU565 human breast adenocarcinoma cells; HEK293 cells and NIH/3T3 mouse embryonic fibroblasts. MDA-MB-231-HER2 cells (a MDA-MB-231 variant that stably overexpresses HER2) and 231/LM2-4 cells were kindly donated by Dr. Kerbel (University of Toronto, Canada). They were all authenticated by STR profiling (Genomics core facility at "Alberto Sols" Biomedical Research institute, Madrid, Spain). They were all maintained at 37 ºC in an atmosphere of 5% CO_2 and cultured as indicated in Table MM1.

3. Genetic knock-down

To stably knockdown CB2R, lentiviral particles containing 3 target-specific shRNA constructs were used. A scrambled shRNA construct was used as control (Santa Cruz Biotechnology). Stably infected cells were selected with puromycin.

For transient knock-down, a pool of double-stranded siRNA duplexes for human ELK1 (Thermo Scientific, Waltham, MA) were used. To knock down c-CBL, human siRNA was purchased from Dharmacon (Lafayette, CO) as a SMARTpool. These reagents combine four SMARTselection-designed siRNAs into a single pool, which guarantees an efficiency of silencing of at least 75%. The non-targeted control siRNA was from Applied Biosystems-Ambion (Austin, TX). DharmaFECT 1 Transfection reagent (Dharmacon) was used as transfection reagent. All the siRNA sequences are in Table MM2.

4. Expression vectors

The expression vectors used in this Thesis were: pLNCX-cSRC K296R/Y528F [dominant negative (Millipore Iberica, Madrid, Spain)], pcDNA3-HA-hCB2R (University of Missouri-Rolla cDNA Resource Center, Rolla, MO), pcDNA3-hERBB2 (kindly donated by Dr. Pandiella, Cancer Research Center, Salamanca, Spain), or the corresponding empty vector, pcDNA3 (Invitrogen, Barcelona, Spain). Fugene HD (Promega, Madison, WI) was used as transfection reagent for overexpression experiments in human breast adenocarcinoma cell lines, and PolyEthylenImine (PEI) (Sigma-Aldrich, San Louis, MO) for transfection in HEK293 cells.

5. Cell viability assays

Cells were seeded at a density of 5000/cm^2 in 10% FBS-containing medium. Twenty four hours later, they were serum starved overnight and then treated with the indicated compounds for 24h. Cells were then stained with crystal violet solution (0.1% crystal violet, 20% methanol in H_2O) for 20 min. After intensive washing with water, the stained cells were solubilized in methanol and absorbance was measured at 570nm, which is proportional to the amount of viable cells in the culture.

6. Anchorage-independent growth assays

Cells were suspended in DMEM supplemented with 10% FBS and 0.35% agar, layered on top of a solid 0.5% agar base in 6-well plates, and incubated at 37°C and 5% CO_2 for 15-45 days. The resulting colonies were morphologically assessed and quantified after staining with crystal violet.

7. Cell invasion assays

Cell invasion was monitored by using Cell Culture Matrigel Invasion Chambers (BD Biosciences, Bedford, MA). Briefly, cells were trypsinized, washed, resuspended in DMEM with 0.1% FBS, and loaded into the upper compartment of the invasion chamber. DMEM supplemented with 10% FBS was placed in the lower compartment as a cell-migration stimulus. Cells were allowed to migrate for 24h at 37ºC through the membrane included in the matrigel invasion chamber. Cell invasion was quantified by staining migrated cell nuclei with DAPI (Invitrogen).

8. Reverse transcription-PCR (RT-PCR) and real-time quantitative–PCR (Q-PCR)

RNA was isolated with Trizol Reagent (Invitrogen), following manufacter´s instructions, and cDNA was obtained with Transcriptor Reverse Transcriptase (Roche Applied Science, Barcelona, Spain). The primers used for RT– and Q–PCR are in Table MM3. For RT–PCR, GAPDH was used as reference. For Q–PCR, probes were from the Universal Probe Library (Roche Applied Science) and 18S, ACTB, GUSB and TBP RNA levels were used as reference.

9. Chromatin immunoprecipitation (ChIP) assays

Cells were processed as recommended by the manufacturer (EZ-ChIP, Millipore, Burlington, MA), and immunoprecipitation was performed with an anti-ELK1 antibody (Table MM4) or a non-specific rabbit IgG as control. ELK1-bound DNA was determined by RT-PCR analysis. Primers for the amplification of ELK1-binding DNA sequences in the CB2R promoter region are in Table MM3.

10. Gene promoter activity assays

A DNA fragment from the immediate upstream region of the CB2R gene, *CNR2* (-614 to +243) was amplified by PCR using human genomic DNA as template and the primers indicated in Table MM3. The 0.85-kb PCR product was cloned using the StrataClone PCR Cloning Kit (Agilent Technologies España, Madrid, Spain) and sequenced with an ABI 3730xl sequencer using T7 reverse and T3

forward primers. Then, it was subcloned into the pGL3 luciferase reporting vector (Promega, Madison, WI) using XmaI (Thermo Scientific). To generate the pGL3-CB2R promoter plasmids containing point mutations in the predicted ELK1-binding sites, QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used with the primers indicated in Table MM3. HEK293 cells were transiently transfected with the following plasmids: pGL3-hCB2R or pGL3-hCB2R mutated plasmids in combination with pCMV5-ELK1-HA plasmid (kindly provided by Dr. Sharrocks, University of Manchester, UK); a constitutively active ELK1 construct (Ser383Asp; Ser389Asp; generated with a QuikChange Site-Directed Mutagenesis Kit; Stratagene); or an empty vector. Transcriptional promoter-driven luciferase activity was analyzed with the Dual-Luciferase Reporter Promega Assay System (Promega) in a Lumat LB9507 luminometer. Renilla-derived luciferase activity was used as internal transfection control.

11. Western blot analysis

Cells and tumors were lysed on RIPA buffer supplemented with 1mM sodium orthovanadate, 0.1mM PMSF, 2μg/μL aprotinin and 2μg/μL leupeptin. Total lysates were resolved by SDS-PAGE, and electrophoretically transferred to PVDF membranes. After blocking with 5% w/v non-fat dry milk in TBST, membranes were incubated with the antibodies indicated in Table MM4. Secondary antibodies were chosen according to the species of origin of the primary antibodies and detected by enhanced chemiluminescence system (Bio-Rad, Hercules, CA). Densitometric analysis was performed with ImageJ software.

12. Co-immunoprecipitation assays

HCC1954 and BT474 cells were transiently transfected with expression vectors pcDNA3-HA-hCB2R and pcDNA3 empty vector, using Fugene HD Transfection Reagent (Promega). HEK293 cells were transiently co-transfected with pcDNA3-hERBB2 and pcDNA3-HA-hCB2R full length or the different CB2R transmembrane constructs (pcDNA3-HA-TMCB2R) (see below), using PEI as transfection agent (Sigma). Forty-eight hours after transfection, cells were lysed on a buffer containing 40mM Hepes pH 7.5, 120mM NaCl, 1mM EDTA, 10mM sodium pyrophosphate, 10mM sodium glycerophosphate, 50mM sodium fluoride, 0.5mM sodium orthovanadate, 0.3% CHAPS, and suplemented with 1mM benzamidine and 0.1mM PMSF. Cell lysates (1mg) were incubated with 5μg of anti-HER2 or anti-HA antibody (Table MM4) covalently coupled to 5μL protein G-sepharose (GE Healthcare, Uppsala, Sweden) overnight at 5ºC on a rotating wheel. Immunoprecipitates were washed with lysis buffer and Hepes buffer (25mM Hepes pH 7.5 and 50mM KCl), resuspended in sample buffer, and filtered through a 0.22μm-pore size Spin-X filter (Sigma-Aldrich). 2-Mercaptoethanol was then added to a concentration of 1% (v/v), and samples resolved by SDS-PAGE and transferred to PVDF. Membranes were blotted with anti-HA or anti-HER2 antibodies.

13. Ubiquitination assays

Cells were lysed after 4h of THC or DMSO treatment using RIPA buffer supplemented with 1mM

sodium orthovanadate, 0.1mM PMSF and 20mM NEM. Cell lysates (1mg) were immunoprecipitated with anti-HER2 antibody (Table MM4) or pre-immune IgG overnight at 4ºC on a rotating wheel. Cell lysates were then incubated with 30µL of protein G-sepharose for 2h at 4ºC on a rotating wheel, and subsequently washed with RIPA buffer lysis. Immunoprecipitates were resuspended in 20µL of sample buffer containing 2-mercaptoethanol. Samples were then resolved and electrophoretically transferred to PVDF membranes as described above, and blotted with anti-ubiquitin antibody (Table MM4).

14. Phosphoarray analyses

A Phospho-Kinase Protein Array (R&D Systems, Abingdon, UK) was used for the simultaneous analysis of 43 different phosphorylation sites by a Western blot-based technique. Cell lysates were incubated with the nitrocellulose membranes provided by the phospho-array. The array was washed to remove unbound proteins, and incubated with biotinylated detection antibodies. Membranes were then incubated with streptavidin-HRP and chemoluminiscence reagents. The densitometric analysis was performed with Image J software.

15. Immunofluorescence analyses

Tissue-Tek or paraffin-embedded tissue sections were fixed in 4% PFA and subjected to heat-induced antigen retrieval in citrate buffer before exposure to the primary antibodies indicated in Table MM4. Cell nuclei were stained with DAPI (Invitrogen). Fluorescence confocal images were acquired by using Leica TCS-SP2 software (Leica, Wetzlar, Germany). Immunofluorescence analysis of CB2R was performed in mammary gland sections of wild type C57Bl/6 mice at different time points of the mammary gland development as previously described (Pisanti and Bifulco 2009).

16. Immunohistochemical analyses

Tissue sections were subjected to a heat-induced antigen retrieval step prior to exposure to an anti-CB2R or an anti-HER2 primary antibody (Table MM4). Immunodetection was performed using the Envision method with DAB as the chromogen. To quantify CB2R expression in the TMAs (see below), cases were scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (high staining). HER2 staining was scored by one pathologist from each institution where the tumor tissue was collected in accordance with HercepTest manufacturer's guidelines.

17. In situ proximity ligation assays

For visualization of endogenous protein-protein interactions, proximity ligation assays (PLAs) were used (Fredriksson *et al.* 2002). For PLAs in human samples and patient-derived xenografts (PDX, see below), sections were deparaffinized and submitted to heat-induced antigen retrieval in sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0). TMA-, PDX- and xenograft-derived slices were permeabilized with PBS containing 0.01% Triton X-100. For PLAs in cell cultures, cells were seeded on glass coverslips at 5000/cm^2. After overnight serum starvation, cells were treated for

4h with THC, TAT-TM peptides (4 μM, see below) or the corresponding vehicle. They were then fixed in 4% paraformaldehyde (PFA), and permeabilized with 0.05% Triton X-100. In all cases, heteromers were detected by using the Duolink *in situ* PLA detection kit (Sigma-Aldrich) following manufacturer's instructions. Briefly, DNA plus and minus oligonucleotides are conjugated to anti-receptor antibodies (proximity probes). This provides a template for ligation, circularization and amplification in the presence of fluorescent nucleotides. This whole process only takes place if the proximity probes are within sufficient close proximity (30-40nm), and can be detected as a fluorescent dotted signal (Söderberg *et al.* 2006). For detection of HER2-CB2R heteromers, cells were incubated with equal amounts of a rabbit anti-CB2 receptor antibody (Table MM4) directly linked to a plus PLA probe, and a rabbit anti-HER2 antibody directly linked to a minus PLA probe. For detection of other HER2-containing heteromers, cells were incubated with a mixture of equal amounts of a mouse anti-HER2 antibody and rabbit anti-HER1 antibody (for HER2-HER1 detection), or a rabbit anti-HER3 antibody (for HER2-HER3 detection). A plus anti-rabbit PLA probe and a minus anti-mouse PLA probe were used. For negative controls, one of the primary antibodies was omitted. Ligation and amplification was done with *In Situ* Detection Reagent Red (Sigma-Aldrich) and slices were mounted in DAPI-containing mounting medium. Samples were analyzed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) and processed with Image J software. Heteromer expression was calculated as the number of red fluorescence dots / total cells in the field. Representative images for each condition were prepared for figure presentation by applying brightness and contrast adjustments uniformly using Adobe Photoshop CS5.

18. Fusion proteins

Sequences encoding amino acid residues 1-155 and 156-238 of YFP Venus protein were subcloned in pcDNA3.1 vector to obtain YFP Venus hemitruncated proteins. The human cDNAs for HER2, CB2R, D44 dopamine receptor and Grelin-1aR, cloned into pcDNA3.1, were amplified without their stop codons using sense and antisense primers harboring EcoRI and BamHI sites (to clone CB2R and Grelin1Ar), XhoI and EcoRI (to clone D44R) or NheI and XhoI (to clone HER2). The amplified fragments were subcloned to be in frame with restriction sites of pRLuc-N1 (PerkinElmer, Wellesley, MA) or pEYFP-N1 (enhanced yellow variant of GFP; Clontech, Heidelberg, Germany) vectors, to generate plasmids that express proteins fused to Rluc or YFP on the C-terminal end (HER2-Rluc, D44R-Rluc, HER2-YFP, CB2R-YFP, or GHS-R1a-YFP). For BiFC experiments (see below), the cDNAs for HER2, CB2R and D44R were also subcloned into pcDNA3.1-nVenus or pcDNA3.1-cVenus to generate a plasmid that expresses the receptor fused to the hemitruncated nYFP Venus or hemitruncated cYFP Venus on the C-terminal end of the receptor (HER2-nVenus, D44-nVenus, CB2R-cVenus).

19. Bioluminiscence resonance energy transfer

HEK-293 cells were transiently co-transfected with a constant amount of a cDNA encoding HER2 or D44R fused to a Rluc protein (HER2-Rluc, D44R-Rluc) as BRET donor, and with increasing amounts of

cDNA of the other receptor fused to YFP (CB2R-YFP, HER2-YFP, GHS-R1a-YFP) as BRET acceptor. For quantification of protein-YFP expression, fluorescence at 530nm was analyzed in a Fluo Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany). Fluorescence of cells expressing the BRET donor only was subtracted from these measurements. BRET signal was analyzed 1 min after addition of 5μM coelenterazine H (Molecular Probes, Eugene, OR) with a Mithras LB 940. To quantify protein-Rluc expression, luminescence was determined 10min after addition of 5μM coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the Rluc construct expressed alone in the same experiment. BRET is expressed as milli BRET units (mBU; net BRET x 1,000). In BRET curves, BRET was expressed as a function of the ratio between fluorescence and luminescence x 100 (YFP/Rluc). To calculate maximum BRET ($BRET_{max}$) from saturation curves, data were fitted using a non-linear regression equation and assuming a single phase with GraphPad Prism software (San Diego, CA).

20. CB_2R transmembrane mutants

A pCDNA3-HA-CB2R plasmid was used as template for the generation of seven mutants containing a HA tag, followed by the N-terminal domain, one transmembrane domain, and the C-terminal domain of CB2R. The primers used to generate these constructs are shown in Table MM5.

21. HIV TAT-TM peptides

Peptides containing the amino acid sequence of CB2R and D44R transmembrane (TM) domains 5 were used as heteromer disrupting agents. To allow intracellular delivery and the correct membrane orientation, they were fused (at the C-terminus domain) to the cell-penetrating HIV TAT peptide. The resulting TAT-TM peptides were:

TM5-TAT CB2R: DYLLSWLLFIAFLFSGIIYTYGHVLWYGRKKRRQRRR

TM5-TAT D44R: YVVYSSVCSFFLPCPLMLLLYWATFYGRKKRRQRRR

They were synthetized at the Peptide Synthesis Facility at University Pompeu Fabra (Barcelona, Spain).

22. Fluorescence complementation assays

HEK293 cells co-transfected with HER2 fused to the YFP Venus N-terminal (n-YFP) and CB2R fused to the YFP Venus C-terminal (c-YFP) were treated with vehicle or the indicated TAT-TM peptides (4μM) for 4h at 37 °C. Fluorescence at 530nm (which only appears after YFP complementation due to proximity of the two receptors fused to cYFP and nYFP hemiproteins) was quantified in a Fluo Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany). Protein complementation was determined as fluorescence of the sample minus fluorescence of non-transfected cells. Cells expressing HER2-nVenus and nVenus or CB2R-cVenus and cVenus showed similar fluorescence levels to non-transfected cells.

23. β-Arrestin recruitment assays

β-Arrestin recruitment was determined by Bioluminescence Resonance Energy Technique (BRET). HEK293 cells were transiently co-transfected with the BRET donor (β-arrestin-1-Rluc, or β-arrestin-2-Rluc) and the BRET aceptor (CB2R-YFP, or HER2-YFP, or CB2R-YFP/HER2 untagged). Forty-eight hours after transfection, the cell suspension was platted in a 96-well microplate (20μg total protein/ well) and stimulated for 7min with the THC agonist (2.5, 5 and 10μM) after addition of coelenterazine H (5μM). BRET readings were collected using a Mithras LB 940 and analyzed as explained before (see BRET methods).

24. Antibody-capture [^{35}S]GTPγS scintillation proximity assays

Specific activation of different subtypes of G_α-proteins by THC (5μM) was determined as previously described (Diez-Alarcia *et al.*, 2016). Briefly, cell membrane homogenates from the four different cell lines [HEK293 cells transiently overexpressing HER2, CB2R, both receptors (HER2-CB2R) simultaneously or the corresponding empty vector (pcDNA3)] were incubated in 96-well Isoplates (Perkin Elmer Life Sciences, Waltham, MA) in incubation buffer containing 0.4nM [^{35}S] GTPγS (Perkin Elmer) and 50 or 100 μM GDP for G_{i2}, $G_{q/11}$ and G_o, or for G_{i1},G_{i3}, G_z, G_s and $G_{12/13}$ proteins, respectively. Specific antibodies for each Gα subunit (mouse monoclonal anti-Gαi1 and anti-Gαo, and rabbit polyclonal anti-Gαi2, anti-Gαi3, anti-Gαz, anti-Gαq/11, anti-Gαs, and anti-Gα12/13; Santa Cruz Biotechnologies) and PVT SPA beads coated with protein A (Perkin Elmer) were used. Radioactivity was quantified on a MicroBeta TriLux scintillation counter (Perkin Elmer).

25. Quantification of anandamide levels

Tissue samples were weighted and homogenized in chloroform:methanol:Tris HCl 50mM (pH 7.5) [2:1:1 (v:v:v)]. Anandamide-d8 (AEA-8) (Cayman Chemical, Ann Arbor, MI) was added as internal standard. The organic and aqueous phases were separated by centrifugation and the organic layer transferred to a clean vial and dried under a stream of argon. The resulting fraction was reconstituted in acetonitrile and analyzed by high-pressure liquid chromatography coupled to mass spectrometry (LC-MS). LC-MS analysis was performed using an Agilent 1200LC-MSD VL instrument. LC separation was achieved with a Zorbax Eclipse Plus C18 column (5μm, 4.6mm x 50mm) together with a guard column (5μm, 4.6mm x 12.5mm). The gradient elution mobile phases consisted of A (95:5 water:acetonitrile) and B (95:5 acetonitrile:water), with 0.1% formic acid as the solvent modifier. The gradient (flow rate of 0.5 mL/min) started at 0% B (for 5min), increased linearly to 100% B over the course of 45min, and decreased to 0% B for 10min before equilibrating for 5min with an isocratic gradient of 0% B. MS analysis was performed with an electrospray ionization source. LC-MS measurements were made by selected ion monitoring in positive mode. Fractions were quantified by measuring the area under the peak and normalized using AEA-8 as internal standard and absolute AEA levels were estimated by comparison with the respective deuterated standard.

26. Animals and drug treatments

All procedures involving animals were performed with the approval of the Complutense University Animal Experimentation Committee and Madrid Regional Government according to the EU official regulations.

a) Generation of MMTV-neu:CB2R $^{-/-}$ mice and sample collection

Generation of the congenic strain MMTV-neu:CB2R-/- was accomplished by mating MMTV-neu mice (The Jackson Laboratory, Bar Harbor, Maine) with CB2R-/- mice (NIH, Bethesda, Maryland). To transfer the CB2R line (with a C57BL/6J background) to the genetic background of the tumor-prone animals (FVB/NJ), the descendants were backcrossed with MMTV-neu mice for 6 generations, using a marker-assisted selection protocol (MASP). In short, the offsprings of each generation were genotyped for CB2R . Heterozygous animals were then genotyped for a set of 377 single nucleotide polymorphisms (SNPs) using the Mouse Low Density Linkage Panel from Illumina®. Selected breeders for the next generation were those with the highest percentage of FVB/NJ-linked SNPs. After 6 backcrosses, animals presented more than 99% FVB/NJ background. CB2R$^{+/-}$ mice from this generation were then crossed between them to generate MMTV-neu: CB2R$^{-/-}$ and their corresponding control littermates (MMTV-neu: CB2R$^{+/+}$). A total of 67 MMTV-neu: CB2R$^{+/+}$ and 42 MMTV-neu: CB2R $^{-/-}$ female mice were analyzed. Females were palpated twice weekly for mammary gland nodules. As soon as tumors appeared, they were routinely measured with external caliper, and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$. Animals were sacrificed and mammary glands, breast tumors, and lungs were collected at the following time points: 1) when the first tumor in each animal appeared, 2) 40 days after the appearance of the first tumor, and 3) 90 days after the appearance of the first tumor. After animal sacrifice, mammary glands and lungs were fixed in 4% PFA. Before PFA fixation, lungs were visually analyzed for macroscopic metastases. Microscopic metastases were determined by H&E staining of PFA-fixed paraffin-embedded sections. Tumors were divided in four portions for 1) preparation of tissue sections for immunofluorescent staining [frozen in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands)], 2) preparation of tissue sections for H&E staining (fixed in buffered 4% PFA), 3) protein extraction (snap frozen), and 4) RNA isolation (snap frozen), and were stored at -80ºC until analysis (except PFA-fixed tumor fractions, that were kept at room temperature).

b) Generation of xenografts and drug treatments

For the generation of orthotopic tumors, $5x10^6$ viable cells were injected into the fourth right mammary fat pad of anesthetized (with 4% isoflurane) 6 week-old SCID female mice (Harlan Interfauna Iberica, Barcelona, Spain). To evaluate the role of CB2R in tumor growth, tumor volume was routinely measured in animals injected with control or CB2R-silenced cells. Fifty days after tumor detection, animals were sacrificed and tumors were collected and processed as described above. To study the

effect of THC, tumor volume was routinely measured with and external caliper, and when it reached an average volume of 200mm^3, animals were randomly assigned to the different groups: THC (1.5mg/animal/dosis) or sesame oil as vehicle. Treatments were administered by oral gavage in 100µL, 3 times a week for 1 month. At the end of the treatment, animals were sacrificed and tumors and organs were collected. Tumors were divided in portions for preparation of tissue sections for PLA staining (frozen in Tissue-Tek) and protein extraction (snap frozen), and were stored at -80°C until analysis.

c) Generation of lung metastases

5x10^5 231/LM2-4 luciferase-expressing cells [a lung-seeking metastatic variant of the MDA-MB-231-HER2 cell line (Munoz *et al.* 2006)] were injected into the lateral tail vein of 6 week-old SCID female mice. Forty five days after cell injection, animals were analyzed by bioluminescence in an IVIS 2000 system (Xenogen Corp, Alameda, CA). Imaging data were processed with Living Image software (Xenogen Corp). Mice were then sacrificed and lungs were collected for metastatic nodule quantification by H&E staining.

27. Patient-derived xenografts

Human breast tumors used to establish patient-derived xenografts (PDX) were from biopsies or surgical resections at Vall d'Hebron University Hospital (Barcelona, Spain), and were obtained following institutional guidelines and approval of the Institutional Review Boards (IRB) at Vall d'Hebron Hospital in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients who provided tissue. Fragments of patient samples were implanted into the mammary fat pad of NOD.CB17-Prkdcscid (NOD/SCID) (#SM-NOD-5S-F, Janvier, France) and maintained with 17 ß-estradiol (1µM) (#E8875-1G, Sigma) in the drinking water. Mice were maintained and treated in accordance with institutional guidelines of Vall d'Hebron University Hospital Care and Use Committee.

28. Tissue microarrays

PFA-fixed and paraffin-embedded blocks of tumor tissue from cases operated in the University Hospitals of Kiel, Tubingen, or Freiburg between 1997 and 2010 were used for tissue microarray (TMA #1 and TMA#2) construction. All patients gave informed consent, and the study was authorized by the respective Hospital Ethics Committees. This resulted in two series of 166 and 483 tumor samples. Complete histopathological information was available for all the patients. Additionally, for the 483-sample series (TMA #2), date and cause of death as well as date of local and/or distant relapse were also available. TMA #3 consisted of 57 samples corresponding to newly diagnosed HER2+ breast cancer patients operated at 12 de Octubre University Hospital (Madrid, Spain) between 1999 and 2013, and prior any treatment. TMA #4 was previously described in (Hergueta-Redondo *et al.* 2016), and contained 138 high-grade ductal breast cancer samples obtained before treatment at the Vall d´Hebron Hospital (Barcelona, Spain), Virgen del Rocío Hospital (Seville, Spain), and MD Anderson Cancer Center (Madrid, Spain), between 2003 and 2014. Of them, 39 corresponded

to HER2+ cases. In all cases, PFA-fixed and paraffin-embedded blocks of tumor tissue were used to generate the corresponding TMAs by punching two 1-mm spots of each patient's biopsy.

29. Analysis of published microarray datasets

CB1R and CB2R mRNA expression in mouse mammary tissue was analyzed in the dataset published in (Clarkson and Watson 2003). Human CB2R mRNA expression for survival correlation studies was obtained from microarray datasets published in (Bild *et al.* 2006; Chin *et al.* 2006; Györffy *et al.* 2010). These arrays contain confirmed HER2 status (amplification as determined by FISH or immunohistochemistry) and survival of the patients. For CB2R-ELK1 correlation analysis, human CB2R and ELK1 mRNA expression were obtained from microarray datasets published in (Bild *et al.* 2006; Chin *et al.* 2006; Desmedt *et al.* 2007; Hatzis *et al.* 2011; Loi *et al.* 2007; Miller *et al.* 2005; Pawitan *et al.* 2005) (Minn *et al.* 2005). The combined raw gene expression of the first 6 micro array datasets was obtained from the "R2: microarray analysis and visualization platform" (http://r2.amc.nl), specifically from the Tumor Breast compendium -Halfwerk-947-complex-u133a.

30. Statistical Analyses

The Pearson's chi-squared test was used for statistical analysis of the human samples included in the TMAs. Kaplan-Meier survival curves were statistically compared by the log-rank test. Unpaired, independent groups of 2 were analyzed by 2-tailed Student's t test. When multi-group comparison was required, data were analyzed by 1-way ANOVA with Tukey's post hoc test, or by 2-way-ANOVA when required. A p value of less than 0.05 was considered statistically significant. Unless otherwise stated, data are expressed as mean ± SEM. All analyses were carried out using GraphPad software, Inc.

Table MM1. Cell lines used and their culture conditions. All growth mediums were supplemented with 10% FBS and 1% penicillin / streptomycin.

Cell lines	Cellular type	Receptor expression	Growth medium
MDA-MB-231	Breast cancer	ER-PR-HER2-	DMEM
MDA-MB-231-HER2	Breast cancer	ER-PR-HER2+	DMEM
231/LM2-4	Breast cancer	ER-PR-HER2+	DMEM
MDA-MB-468	Breast cancer	ER-PR-HER2-	DMEM
MCF7	Breast cancer	ER+PR+HER2-	MEM + 10µg/mL insulin
T47D	Breast cancer	ER+PR+HER2-	RPMI
BT474	Breast cancer	ER+PR+HER2+	RPMI + 10µg/mL insulin
MDA-MB-361	Breast cancer	ER+PR-HER2+	L-15
SKBR3	Breast cancer	ER-PR-HER2+	McCoy´s 5A
HCC1954	Breast cancer	ER-PR-HER2+	RPMI
AU565	Breast cancer	ER-PR-HER2+	RPMI
HEK293	Embryonic kidney	-	DMEM
NIH/3T3	Mouse embryonic fibroblasts	-	DMEM

Table MM2. siRNA sequences used for transient knock down.

Pool siRNA	ELK1	c-CBL
1	5'-GCAAGGCAAUGGCCACAUC-3'	5'-AAUCAACUCUGAACGAAA-3'
2	5'-CGGAAGAGCUUAAUGUGGA-3'	5'-GACAAUCCUCACAAUAAA-3'
3	5'-GCCAGAAGUUCGUCUACAA-3'	5'-UAGCCCACCUUAUAUCUUA-3'
4	5'-GCAGCAGCCGGAACGAGUA-3'	5'-GGAGACAUUUCGGAUUA-3'
Non-targeted siRNA	5'-UUCUCCGAACGUGUCACGU-3'	

Table MM3. Primers used for reverse-transcriptase (RT) PCR, real-time quantitative (Q) PCR and mutagenesis.

RT-PCR primers	**Sense**	**Antisense**
mCB$_2$R	5′-CTCATGGGGTGGACTTGTTG-3′	5′-ACCTTGGGCCTTCTTCTTTC-3′
rERBB2	5′-GCTCAGAGACCTGCTTTGGA-3′	5′-AGGAGGACGAGTCCTTGTAGTG-3′
GAPDH	5′-GGGAAGCTCACTGGCATGGCCTTCC-3′	5′-CATGTGGGCCATGAGGTCCACCAC-3′
CB$_2$R promoter (ChIP)	5′-TGGTAACAGGCACGGAAGGC-3′	5′-TGAGTGCCACCCCAAGCCAG-3′

Q-PCR primers	**Sense**	**Antisense**
mFAAH	5′-GCAGGTGGGCTGTTCAGT-3′	5′-AAGCAGGGATCCACAAAGTC-3′
mTenascin C	5′-GGGCTATAGAACACCGATGC-3′	5′-CATTTAAGTTTCCAATTTCAGGTTC-3′
mSPARC	5′-GGTTGGCACCCACAGGTA-3′	5′-GCCCCTCAGCAGACTGAA-3′
mCOX2	5′-GATGCTCTTCCGAGCTGTG-3′	5′-GGATTGGAACAGCAAGGATTT-3′
mCB$_1$R	5′-GGGCAAATTTCCTTGTAGCA-3′	5′-GGCTCAACGTGACTGAGAAA-3′
mCB$_2$R	5′-CAGCTCTTGGGACCTACGTG-3′	5′-TGTCCCAGAAGACTGGGTGT-3′
hCB$_2$R	5′-TGGGACAGGGTCAGTACAAGT-3′	5′-CTTTGGCTCCTGTGGTCT-3′
hELK1	5′-GCTTCCTACGCATACATTGACC-3′	5′-GGTGCTCCAGAAGTGAATGC-3′
hHER2	5′-GGGAAACCTGGAACTCACCT-3′	5′-CCCTGCACCTCCTGGATA-3′
hACTB	5′-CCAACCGCGAGAAGATGA-3′	5′-CCAGAGGCGTACAGGGATAG-3′
hTBP	5′-CCCATGACTCCATGACC-3′	5′-TTTACAACCAAGATTCACTGTGG-3′
hGUSB	5′-CGCCCTGCCTATCTGTATTC-3′	5′-TCCCCACAGGGAGTGTGTAG-3′
18S	5′-GCTCTAGAATTACCACAGTTATCCAA-3′	5′-AAATCAGTTATGGTTCCTTTGGTC-3′

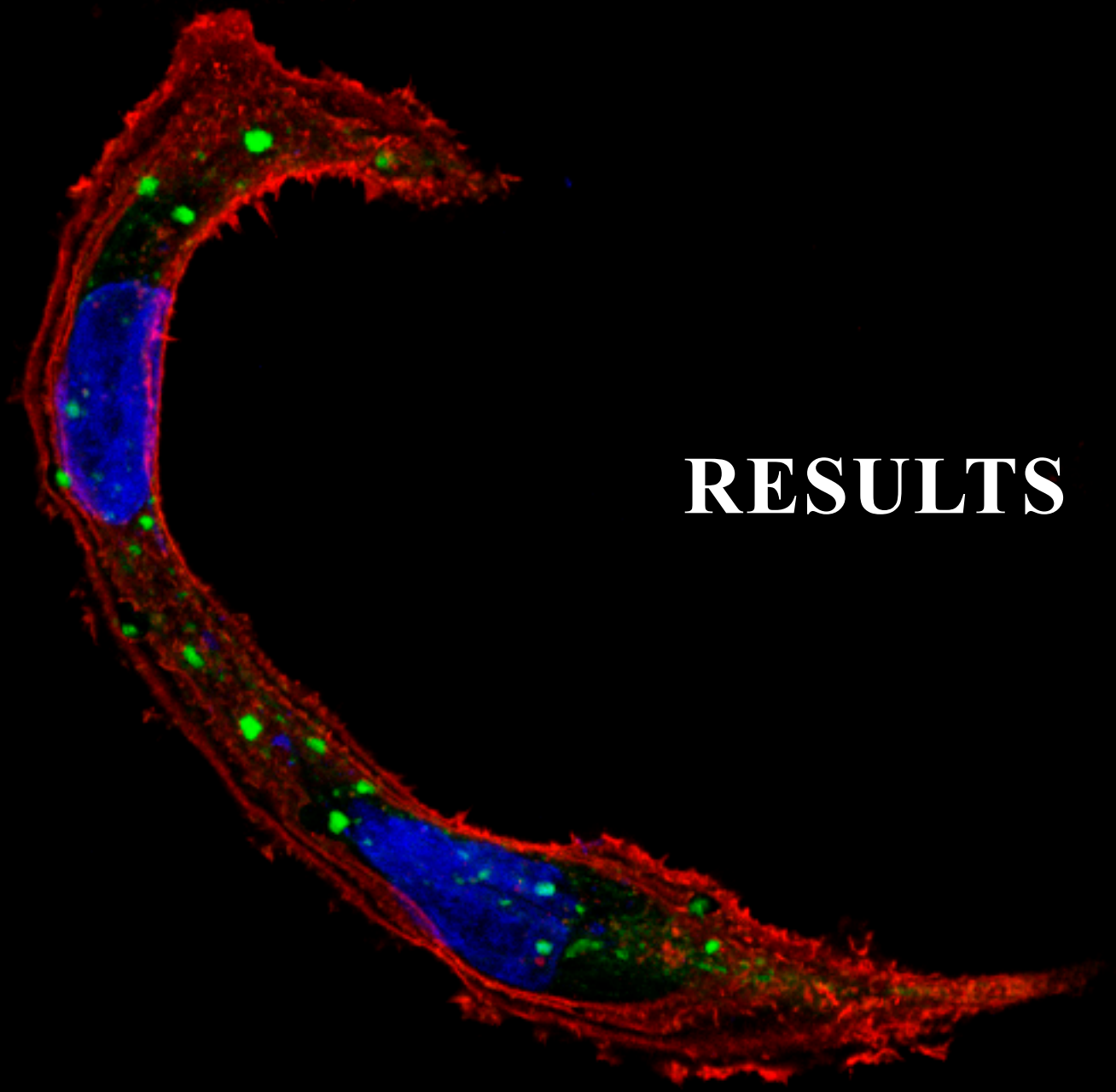
Mutagenesis primers	
(-614 to +243)-hCB$_2$R	**Sense:** 5′-CCCGGGTATACTCCCAAGCAGAAAAG-3′ **Antisense:** 5′-CTCGAGATACAGGTGTTGGGGAATGA-3′
pGL3 (m587)-hCB$_2$R	**Sense:** 5′-CAAGCAGAAAAGCTTGGCTctacCTAAGAGGTACCTGCCCCT-3′ **Antisense:** 5′-AGGGGCAGGTACCTCTTAGgtagAGCCAAGCTTTTCTGCTTG-3′
pGL3 (m404)-hCB$_2$R	**Sense:** 5′-GAGGGGCAGGTACCTCTTAGgcagAGCCAAGCTTTTCTGCTTGG-3′ **Antisense:** 5′-CCAAGCAGAAAAGCTTGGCTctgcCTAAGAGGTACCTGCCCCTC-3′
pGL3 (m325)-hCB$_2$R	**Sense:** 5′-CAAAGCTCTGGCCTAGTGGTGAAGAGGCATTctgcTGGCATGTCCTTTTAG-3′ **Antisense:** 5′-CTAAAAGGACATGCCAgcagAATGCCTCTTCACCACTAGGCCAGAGCTTTG-3′
pGL3 (m244)-hCB$_2$R	**Sense:** 5′-TTACAGATAAAGAAACTTGCCTTTGGcggcGTTAAGTGAATCAACATTTTAACGAG -3′ **Antisense:** 5′-CTCGTTAAAATGTTGATTCACTTAACgccgCCAAAGGCAAGTTTCTTTATCTGTAA-3′
pGL3 (m89)-hCB$_2$R	**Sense:** 5′-CACCAGGGACCTGGAGGctgcGTGGTAACAGGCACGGA-3′ **Antisense:** 5′-TCCGTGCCTGTTACCACgcagCCTCCAGGTCCCTGGTG-3′
pGL3 (m71)-hCB$_2$R	**Sense:** 5′-GGAAGTGGTAACAGGCACctgcGGCCAGACCTCCTCACAC-3′ **Antisense:** 5′-GTGTGAGGAGGTCTGGCCgcagGTGCCTGTTACCACTTCC-3′
ELK1 constitutively active (Ser383Asp)	**Sense:** 5′-TCTGGAGCACCCTGGATCCCATTGCGCCCC-3′ **Antisense:** 5′-GGGGCGCAATGGGATCCAGGGTGCTCCAGA-3′
ELK1 constitutively active (Ser389Asp)	**Sense:** 5′-GATCCCATTGCGCCCCGTGACCCGGCCAAG-3′ **Antisense:** 5′-CTTGGCCGGGTCACGGGGCGCAATGGGATC-3′

Table MM4. Antibodies used for immunohistochemistry (IHC), Western blot (WB), immunofluorescence (IF), chromatin immunoprecipitation (ChIP), co-immunoprecipitation (co-IP) and proximity ligation assays (PLA).

Primary antibodies	Species	Source	Use
anti-α Tubulin	mouse	Sigma-Aldrich	WB
anti-β Actin	mouse	Sigma-Aldrich	WB
anti-AKT	rabbit	Cell Signaling Technology	WB
anti-CB_2R (PA1-744)	rabbit	Thermo Fisher Scientific	IHC, WB, IF
anti-CB_2R	rabbit	Cayman Chemical	PLA
anti-Cyclin D1 (M-20)	rabbit	Santa Cruz Biotechnology	WB
anti-E-Cadherin	mouse	BD Biosciences	IF
anti-ELK1 ChIP Grade antibody	rabbit	Abcam	WB, ChIP
anti-ERK	rabbit	Cell Signaling Technology	WB
anti-HA	rabbit	Cell Signaling Technology	WB
anti-HA (3F10)	rat	Roche Diagnostics	Co-IP
anti-p21 (F-5)	mouse	Santa Cruz Biotechnology	WB
anti-phospho-AKT (Ser473)	rabbit	Cell Signaling Technology	WB
anti-phospho-c-SRC (Tyr 418)	mouse	Millipore	WB, IF
anti-phospho-ELK1 (Ser 383) (cloneB4)	mouse	Santa Cruz Biotechnology	WB, IF
anti-phospho-ERK (Thr202/ Tyr204)	rabbit	Cell Signaling Technology	WB
anti-SRC	rabbit	Santa Cruz Biotechnology	WB
anti-HER1	rabbit	Millipore	WB, PLA
anti-HER2 (C-18)	rabbit	Santa Cruz Biotechnology	WB, co-IP, PLA
anti-HER2	mouse	Cell Signaling Technology	IF, PLA
anti-HER2 (HercepTest)	rabbit	Dako	IHC
Anti-phospho-HER2 (Tyr 1248)	rabbit	Cell Signaling Technology	WB
anti-HER3	rabbit	Cell Signaling Technology	WB, PLA
anti-HER4	rabbit	Cell Signaling Technology	WB
anti-c-CBL	mouse	BD Bioscience	WB
anti-CHIP	mouse	Abcam	WB
anti-Ubiquitin	mouse	Santa Cruz Biotechnology	WB
Secondary antibodies			
anti-mouse IgG–HRP	goat	GE Healthcare	WB
anti-mouse Alexa Fluor 594	goat	Invitrogen	IF
anti-rabbit IgG–HRP	goat	GE Healthcare	WB
anti-rabbit Alexa Fluor 488	goat	Invitrogen	IF

Table MM5. Primers used for CB2R transmembrane mutant constructions.

CB_2R transmembrane mutants	**Sense primers**	**Antisense primers**
HA-PS-CB_2R-Nt	5′-AAACTTAAGCTTGGTACCACC-3′	5′-CAACGAATTCCTTCTGGGGACCACTCAGGATC-3′
CB_2R-TM1	5′AATTCAAGACAGCTGTTGCTGTGTTGTGCACTCTTCTGGGCCTGCTAAGTGCCCTGGAGAACGTGGCTGTGCTCTATCTGATCCTGTCCG-3′	5′GGATCCGGACAGGATCAGATAGAGCACAGCCACGTTCTCCAGGGCACTTAGCAGGCCCAGAAGAGTGCACAACACAGCAACAGCTGTCTTGAATTC-3′
CB_2R-TM2	5′GGATTCAATGTGTTTAGCTGCGCATTTGTCGTGAGTGCCCTGTTCGACGCTGGGGCTTTGAGCGGCATTTTCCTGGGATTC-3′	5′GGATCCCAGGAAAATGCCGCTCAAAGCCCCAGCGTCGAACAGGGCACTCACGACAAATGCGCAGCTAAACACATTGGATTC-3′
CB_2R-TM3	5′GAATTCGCTGTCTTCCTGCTGAAGATTGGCAGCGTGACTATGACCTTCACAGCCTCTGTGGGTAGCCTCCTGCTGACCGCCATTGACGGATTC-3′	5′GGATCCGTCAATGGCGGTCAGCAGGAGGCTACCCACAGAGGCTGTGAAGGTCATAGTCACGCTGCCAATCTTCAGCAGGAAGACAGCGAATTC-3′
CB_2R-TM4	5′GGATTCACTTGGGGAATGCTCCCCCTGTACTCCGTCCTAGCATCACTCGTCTGGATGATCGGCCTGACCGTGCTGGCAAGGGGATTC-3′	5′GGATCCCCTTGCCAGCACGGTCAGGCCGATCATCCAGACGAGTGATGCTAGGACGGAGTACAGGGGGAGCATTCCCCAAGTGAATTC-3′
CB_2R-TM5	5′-CTGGAATTCAATGACTACCTGCTGAGCTGG-3′	5′-GCTGGGATCCCTTCCAGAGAACATGCCCATAG-3′
CB_2R-TM6	5′GGATTCAGCCACGCCATGCTCGCCCTGGTGCCATTCTGGTGTATCCTCCTCGTGGCTTTGGTGCTAGGGCTAACCGGATTC-3′	5′GGATCCGGTTAGCCCTAGCACCAAAGCCACGAGGAGGATACACCAGAATGGCACCAGGGCGAGCATGGCGTGGCTGAATTC-3′
CB_2R-TM7	5′GGATTCAAGGCCTTTGCTTTCTGCTCCATGCTGTGCCTCATCAACTCCATGGTCAACCCTGTCATCTATGCTCTACGGGGATTC-3′	5′GGATCCCCGTAGAGCATAGATGACAGGGTTGACCATGGAGTTGATGAGGCACAGCATGGAGCAGAAAGCAAAGGCCTTGAATTC-3′
CB_2R-Ct	5′-TATGGATCCCGGAGTGGAGAGATCCGCTCCTC-3′	5′-GGAACGGGCCCTCTAGACTCCG-3′



RESULTS

AIM1. ANALYSIS OF THE ROLE OF CB2R IN BREAST CANCER GENERATION AND PROGRESSION

SUMMARY

The ECS has emerged as an essential cell communication system controlling multiple biological functions. Moreover, alterations in this system have been reported in a wide variety of pathologies, including cancer. Specifically, in breast tumors, an overexpression of the CB_2R has been described when compared to healthy tissue (Caffarel *et al.* 2006; Elbaz *et al.* 2016). However, it is not clear whether this alteration is a cause or a consequence of the disease. Hence, the first aim of this Thesis was to investigate the role of CB_2R in breast tumor generation and progression.

First, we confirmed in a large series of human samples that CB_2R is overexpressed in breast cancer with respect to non-cancerous tissue, and observed that this overexpression is highly associated to the HER2+ subtype. Moreover, we found a strong correlation between higher CB_2R protein expression and lower patient survival in this specific subgroup.

To analyze the mechanistic and functional relevance for the link between high CB_2R expression and HER2+ tumor aggressiveness, we used a wide variety of cell- and animal-based experimental approaches. Thus, CB_2R silencing impaired cancer cell proliferation and invasion as well as tumor growth and metastasis in a HER2+ context, whereas it caused no such effects in HER2-negative cells or tumors. In addition, we characterized the molecular mechanism underlying the functional connection between the two receptors, which involves the upregulation of CB_2R by HER2 (*via* activation of the transcription factor ELK1 through the ERK cascade), and the subsequent stimulation of HER2 pro-oncogenic signaling *via* the non-receptor tyrosine kinase c-SRC.

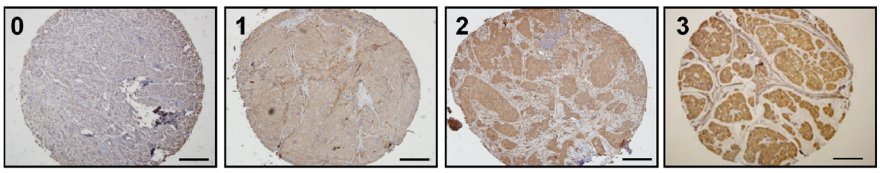
Altogether, our findings shed light on the biological significance of CB_2R deregulation in breast cancer, unmasking a remarkable pro-tumoral role in HER2+ tumors. In addition, our results support that CB_2R is an indispensable component of the HER2 pro-oncogenic signaling machinery.

AIM1. ANALYSIS OF THE ROLE OF CB2R IN BREAST CANCER GENERATION AND PROGRESSION

1.1. Elevated CB2R expression in HER2+ tumors correlates with poor patient prognosis

As explained in the Introduction, previous observations of our laboratory had shown, in two small cohorts of human samples, that CB2R mRNA expression in breast tumors correlates with higher histological grades and increased HER2 expression (Caffarel *et al.* 2006), and that the CB2R protein was present in the vast majority of HER2+ tumors (Caffarel *et al.* 2010). Here, we analyzed CB2R protein expression in a much larger series of tissue sections [649 breast human samples included in different tissue microarrays (TMAs #1 and #2)] in order to confirm and strengthen our previous results. CB2R expression, as determined by immunohistochemistry analysis, was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining) or 3 (high staining) (Figure R1A). We observed that non-tumor breast tissue expressed undetectable levels of CB2R (Figure R1B). Conversely, the receptor was detected in a very large fraction of human breast adenocarcinomas (476 out of 629, *i.e.* 76%). In addition, CB2R expression was highly associated to HER2+ tumors, while no such association was found with hormone-sensitive or triple negative tumors (Figure R1C). Thus, 97% of the HER2+ samples scored positive for CB2R expression and 65% of them expressed elevated levels of the receptor (scores 2 and 3) (Figure R1C).

A



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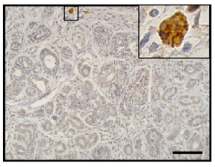


C

		n	CB_2R staining 0	1	2	3	p-value
Hormone receptor status	Positive	504	117 (23.2%)	191 (37.9%)	135 (26.8%)	61 (12.1%)	0.66
	Negative	117	24 (20.5%)	41 (35.0%)	38 (32.5%)	14 (12.0%)	
HER2 status	Positive	92	3 (3.3%)	29 (31.5%)	39 (42.4%)	21 (22.8%)	**<0.001**
	Negative	360	86 (23.9%)	141 (39.2%)	94 (26.1%)	39 (10.8%)	
Triple negative status	Positive	47	13 (27.6%)	20 (42.6%)	12 (25.5%)	2 (4.3%)	0.14
	Negative	405	76 (18.8%)	150 (37.0%)	121 (29.9%)	58 (14.3%)	

Figure R1. CB2R protein expression associates with HER2+ breast tumors. **(A)** Representative images showing CB2R protein expression scoring according to staining intensity in tissue microarray (TMA) samples: scores 0, 1, 2 and 3 correspond to no, low, moderate and high staining, respectively. **(B)** Representative CB2R immunohistochemical staining in a human non-tumor breast tissue sample included in the analyzed TMAs. Inset, CB2R staining (brown) in a macrophage is shown as a positive control of CB2R expression. Scale bar=500μm **(C)** Association between CB2R expression (as determined by staining scoring) and the molecular features of breast tumor samples included in TMAs #1 and #2 . The Pearson's chi-squared test was used for statistical analysis.

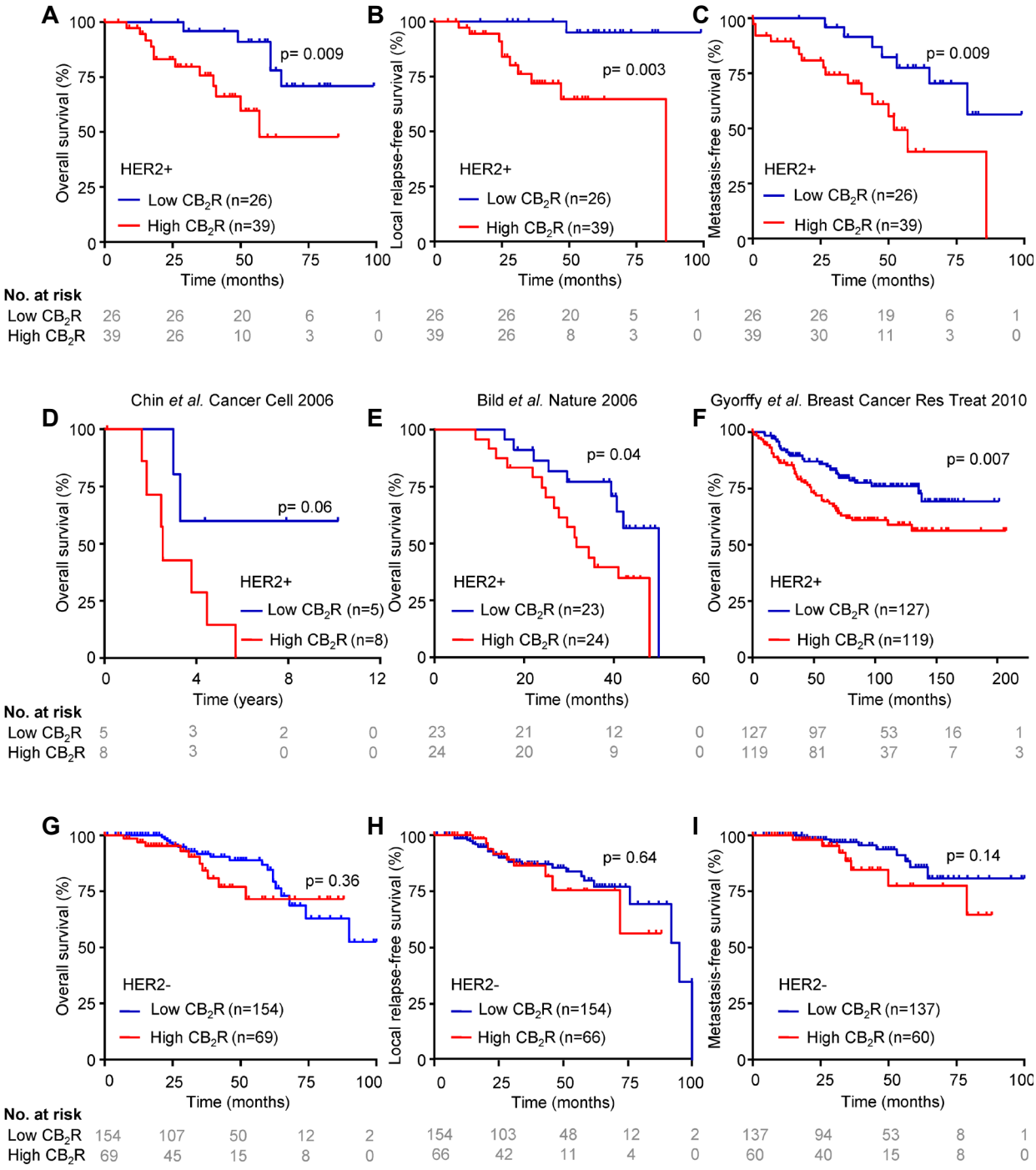


Figure R2. Elevated CB2R expression in HER2+ breast tumors correlates with poor patient prognosis.
Kaplan-Meier curves for overall survival **(A, D-G)**, local relapse-free survival **(B, H)** and metastasis-free survival **(C, I)**. Numbers below x-axes correspond to the number of patients at risk in each group at the indicated time points. Data plotted in panels **(A-C)** correspond to the 65 HER2+ samples included in TMA # 2 (see Methods). Data plotted in panels **(G-I)** correspond to the 224 HER2- samples included in TMA #2. In **(A-C, G-I)** samples were classified by high CB2R expression (IHC scores 2 and 3) and low CB2R expression (IHC scores 0 and 1). Data plotted in panels **(D,E)** were obtained from the microarray data sets published in ArrayExpress database (accession number E-TABM-158) **(D)** and GEO database (accession number GSE3143) **(E)**. Data plotted in **(F)** were obtained from (Gyorffy *et al.* 2010) through the Kaplan-Meier Plotter (www.kmplot.com). In **(D,E)**, samples were ranked by CB2R mRNA expression, and the best cutoff was manually selected. In **(F)**, the best cutoff was automatically selected by the software. Survival curves were statistically compared by the log-rank test. All statistical test were two-sided.

To further analyze the role of CB_2R overexpression in HER2 + breast cancer, we divided the human samples in TMA#2 (the one with complete clinical information) in two different groups based on CB_2R expression [low (scores 0 and 1) *vs* high (scores 2 and 3)] and studied their clinocopathologic characteristics. Patients with HER2+/high CB_2R presented decreased overall survival (Figure R2A), and higher probability to suffer local recurrence (Figure R2B) and to develop distant metastases (Figure R2C) than those with HER2+/low CB_2R tumors. Similar observations were made when CB_2R mRNA levels were analyzed in public DNA microarray datasets (Bild *et al.* 2006; Chin *et al.* 2006; Györffy *et al.* 2010) (Figures R2D-F). Of interest, this correlation was not observed in HER2- patients (Figures R2G-I). Together, these results show a strong association between CB_2R expression and tumor aggressiveness in HER2+ breast cancer.

1.2. Genetic inactivation of CB_2R impairs breast tumor generation and progression

We next analyzed whether there was a cause-to-effect link between elevated CB_2R expression and increased aggressiveness in HER2+ tumors. First, we observed that CB_2R expression in the non-cancerous mammary glands of adult wild-type (WT) female mice was virtually undetectable (Supplementary Figure 1A-B). Moreover, it remained very low and unchanged during adult mammary gland development (Supplementary Figure 1A-B). These results suggest that CB_2R may not play a major role in the physiology of the healthy adult mammary gland. Next, we analyzed breast tumor generation and progression in an animal model of HER2-driven breast cancer (the MMTV-neu mouse) in which CB_2R expression was knocked-out (Supplementary Figure 2A-B). MMTV-neu:$CB_2R^{-/-}$ mice (n=42) showed a striking delay in tumor onset as compared with their WT littermates (n= 67) (Figure R3A). Upon early detection, tumor histological features were very similar in both groups (low grade adenocarcinomas with no lymphatic invasion) (Supplementary Figure 2C), the only apparent difference between them being their mitotic index (medium in WT animals and low in the $CB_2R^{-/-}$ population) (Supplementary Figure 2C). Forty days after their appearance, 100% of the CB_2R KO-derived tumors kept their original histological characteristics, while 40% of those derived from WT animals were solid carcinomas with necrotic areas (Supplementary Figure 2C). At the final stage of the disease (90 days after tumor appearance), $CB_2R^{-/-}$ tumors had negligible changes in their histology (although 40% of them presented necrotic areas), but the WT group included 40% of solid carcinomas, 40% of tumors with necrotic areas and 20% with evident signs of lymphatic invasion (Supplementary Figure 2C), all of them signs of tumor aggressivess.

The lack of CB_2R also reduced the number of tumors generated per animal (Figure R3B) and slowed-down tumor growth (Figure R3C). The delayed tumor onset and the decreased tumor multiplicity and growth associated to the lack of CB_2R were accompanied by reduced levels of Cyclin D1 and increased levels of the CDK inhibitor p21 in the tumors (Figures R3D-E), both of which are hallmarks of HER2-induced malignant transformation and progression (Yu *et al.* 2001; Muraoka *et al.* 2002; Cheng *et al.* 2010). In addition, the levels of the endocannabinoid anandamide were higher in $CB_2R^{-/-}$-derived tumors than in $CB_2R^{+/+}$ tumors (Supplementary Figure 2D), and the mRNA levels

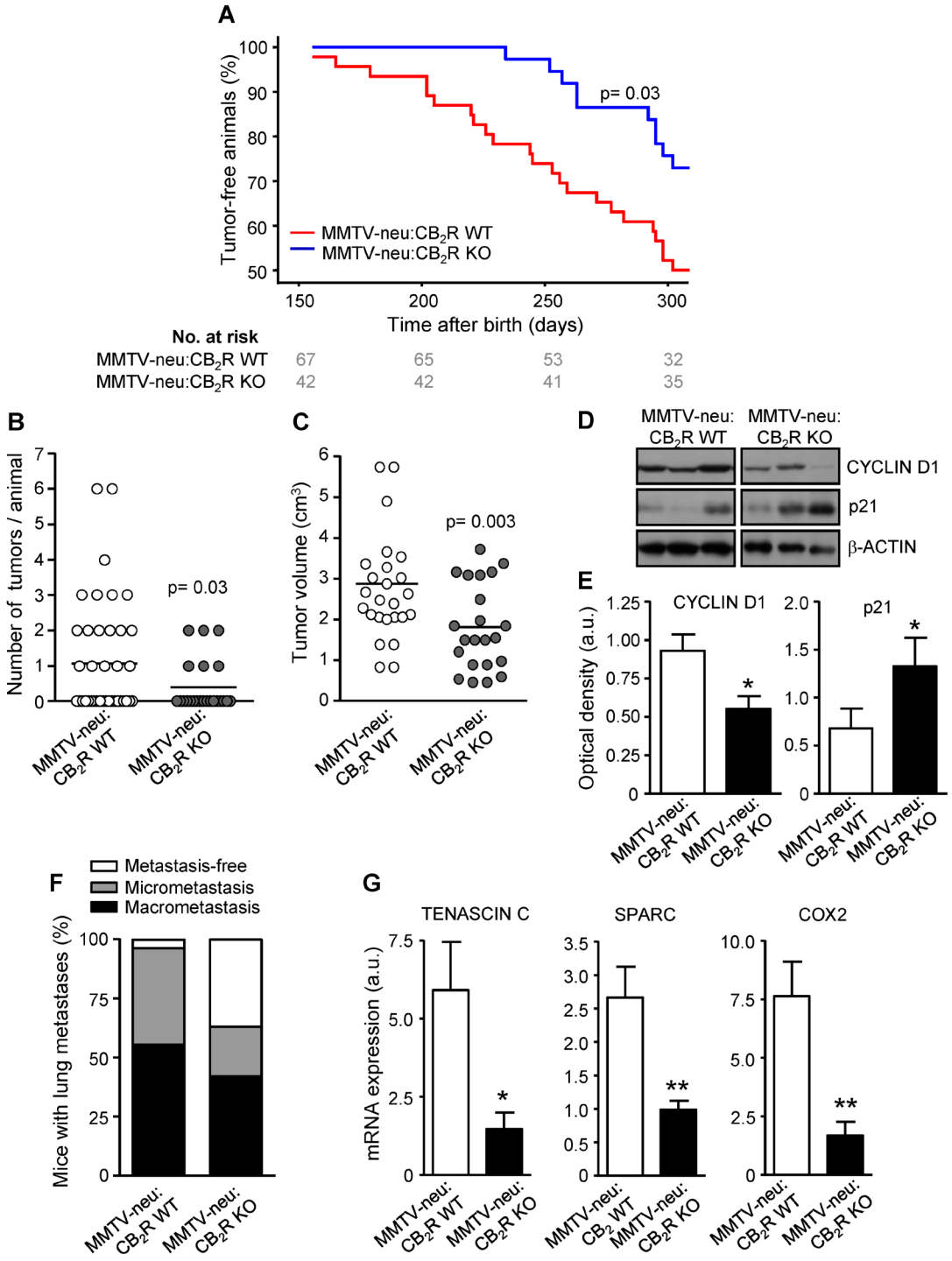


Figure R3. The lack of CB2R impairs breast tumor generation and progression.
(A) Kaplan-Meier curves for tumor onset in MMTV-neu:CB2R WT and MMTV-neu:CB2R KO mice. Numbers below x-axes correspond to the number of mice at risk in each group at the indicated time points. Results were analyzed by the log-rank test. **(B)** Number of tumors generated per animal 90 days after first tumor arousal. **(C)** Tumor volume 70 days after tumor appearance. **(D)** Western blot analysis of Cyclin D1 and p21 in tumors generated by the indicated animals. Three representative samples per experimental group are shown. **(E)** Densitometric analysis of the levels of the indicated proteins (determined by Western blot; n=7 for MMTV-neu:CB2R WT tumors and n=6 for MMTV-neu:CB2R KO tumors). Results are expressed in arbitrary units. **(F)** Percentage of animals with lung metastases 90 days after tumor arousal. Lung tumor masses were classified as macrometastases when they were visible to the naked eye at dissection, and as micrometastases when they were only detectable by hematoxylin and eosin staining. **(G)** mRNA levels (as determined by real-time quantitative PCR) of Tenascin C, SPARC and COX2 in tumors generated by the indicated mice. Results are expressed in arbitrary units. (n = 16 for MMTV-neu:CB2R WT tumors and n = 16 for MMTV-neu:CB2R KO tumors). Except in A, data were analyzed by ANOVA with a post hoc analysis by the Student-Newman-Keuls' test. * $P < 0.05$; ** $P < 0.01$ *vs* MMTV-neu:CB2R WT mice.

of the enzyme responsible for anandamide degradation (FAAH) were lower in CB2R-deficient tumors than in CB2R WT lesions (Supplementary Figure 2E). Finally, CB2R deficiency produced a remarkable reduction in the percentage of animals with lung metastases (Figure R3F). This phenotype correlated with decreased tumor levels of Tenascin-C, SPARC, and COX2 (Figure R3G), which have been proposed to mediate metastasis specifically directed to the lungs (Minn *et al.* 2005). Together, these results indicate that CB2R plays an important role in promoting HER2+ breast tumor generation and progression.

1.3. HER2 enhances CB2R expression by activating the transcription factor ELK1 *via* ERK

Since we observed that virtually all HER2+ human tumors express CB2R (Figure R1), we analyzed whether HER2 controls the expression of the cannabinoid receptor. Ectopic overexpression of HER2 in triple-negative (no ER, PR or HER2 expression) MDA-MB-231 cells resulted in an increased transcription of CB2R (Figure R4A). The *in silico* analysis of the CB2R promoter sequence revealed

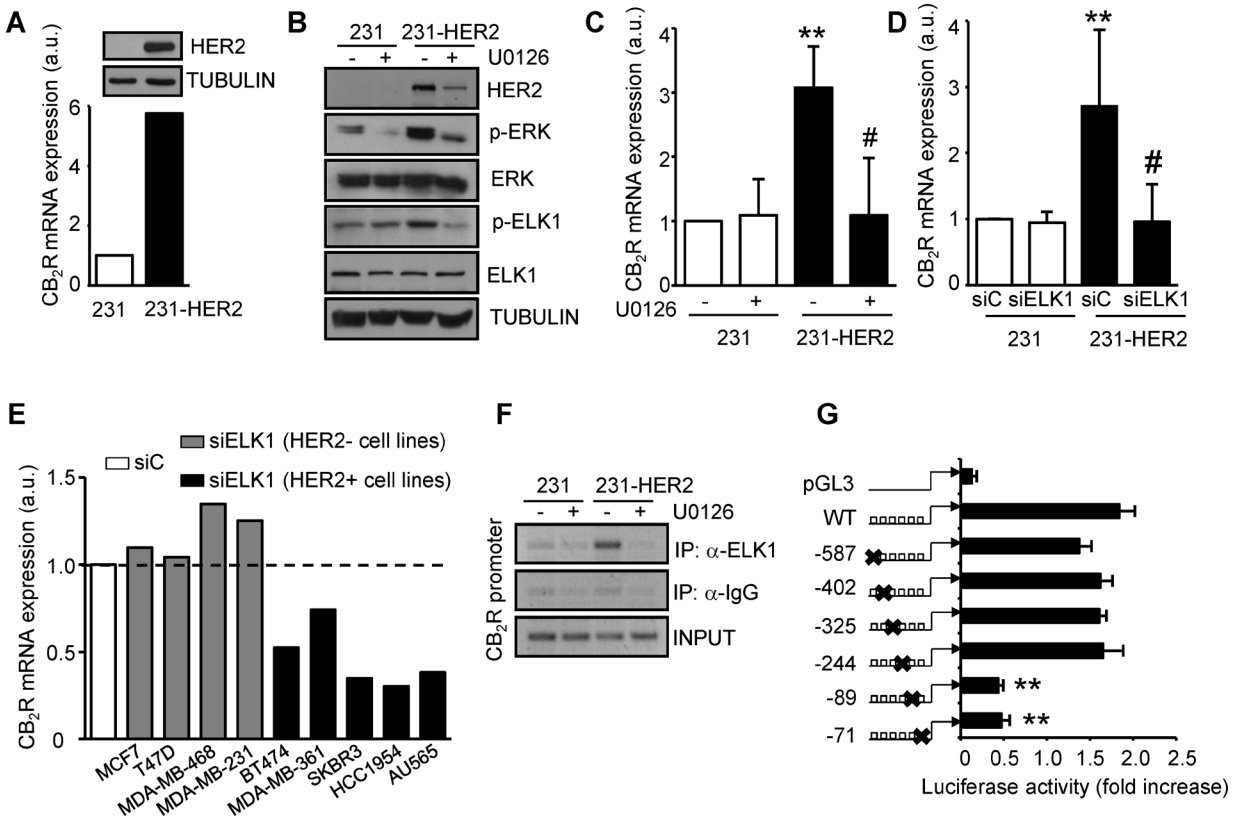


Figure R4. HER2 enhances CB2R expression by activating the transcription factor ELK1 *via* ERK.
(A) HER2 protein expression (upper panel) and CB2R mRNA expression (lower panel) in MDA-MB-231-HER2 (231-HER2) and MDA-MB-231 cells (231). **(B)** Western blot analysis of the indicated proteins, in the presence or in the absence of the MEK inhibitor U0126 (5μM). **(C,D)** CB2R mRNA expression (in arbitrary units), in the presence/absence of U0126 **(C)**, or after transfection with ELK1 siRNAs (siELK1) or with a non-targeted siRNA (siC) **(D)** (n=3 independent experiments). **(E)** Effect of ELK1 knock-down on CB2R mRNA expression in different human breast cancer cells endogenously overexpressing (black bars) or not (gray bars) HER2. Results are expressed in arbitrary units *vs* mRNA expression in the corresponding cells transfected with a control siRNA (siC), which was set at 1 in all cases (white bar). **(F)** ChIP assay in cells treated with or without U0126. Immunoprecipitation was performed with an anti-ELK1 antibody (or a non-specific rabbit IgG as control). **(G)** CB2R gene promoter activity as determined by a luciferase reporter (n=3). Drawings (left) represent the CB2R promoter construct transfected in each case. A constitutively active ELK1 containing plasmid was always co-transfected with the CB2R promoter. Line 1, CB2R promoter empty vector. Data were analyzed by ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test. **, p<0.01 *vs* vehicle-treated **(C)** or siC-transfected **(D)** 231 cells; #, p<0.05 *vs* vehicle-treated **(C)** or siC-transfected **(D)** 231-HER2 cells; **, p<0.01 *vs* WT **(G)**.

the existence of, among others, several ELK1-binding sites (Treisman *et al.* 1992) (Supplementary Figure 3A). ELK1 belongs to the ETS transcription factor family, which has been related to cancer (Seth and Watson 2005), and is a well-established target of the ERK cascade (Janknecht and Hunter 1997). We observed that HER2 overexpression activated ELK1, an effect that was accompanied by the activation of ERK (Figure R4B). Of interest, incubation with the MEK inhibitor U0126 prevented the enhancement of p-ELK1 levels (Figure R4B). Moreover, pharmacological inhibition of MEK (Figure R4C) and genetic knock-down of ELK1 (Figure R4D; Supplementary Figure 3B) blocked the increase in CB2R mRNA levels elicited by HER2 overexpression. Likewise, ELK1 knock-down (Supplementary Figure 3C) decreased CB2R mRNA levels in breast cancer cells that endogenously overexpress HER2, an effect that was not observed in HER2-negative cells (Figure R4E). By chromatin immunoprecitation assays, we confirmed that ELK1 physically interacts with the CB2R promoter, and that this interaction is enhanced upon HER2 overexpression and prevented by inhibition of the ERK cascade (Figure R4F). Moreover, ELK1 was able to activate the CB2R promoter. Thus, transfection of HEK293 cells with a luciferase reporter encoding the CB2R gene promoter (pGL3- CB2R) together with a constitutively active ELK1-expressing plasmid resulted in an increased luciferase activity when compared to cells transfected with pGL3- CB2R only (Figure R4G). Point mutations in the CB2R promoter revealed that the putative ELK1-binding sites located at positions -71 and -89 are the ones responsible for ELK1-induced activation of CB2R expression (Figure R4G). Together, these observations demonstrate that HER2 promotes CB2R upregulation by activating the transcription factor ELK1 *via* ERK activation. Supporting the relevance of this observation, the analysis of 1453 human breast cancer samples from 7 different public DNA microarrays (see Materials & Methods for further details) showed a strong

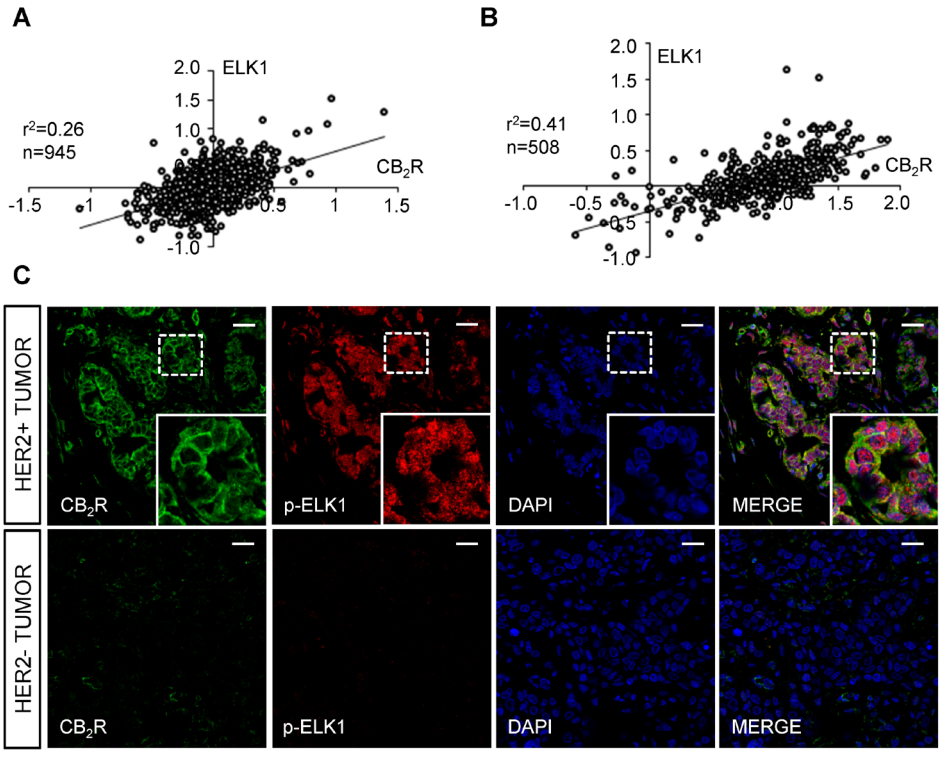


Figure R5. Association between CB2R and ELK1 expression in human breast cancer samples.
(A, B) Correlation of CB2R and ELK1 expression (analyzed by the Pearson's correlation test) in human breast cancer samples from seven public DNA microarrays (see Methods). **(C)** Immunofluorescence analysis of CB2R (green) and phospho-ELK1 (red) in a HER2-positive (upper panels) and a HER2-negative (lower panels) human breast cancer sample. Cell nuclei are stained in blue. Scale bar = 100 μm.

correlation between ELK1 and CB2R mRNA expression (Figures R5A-B), and the immunofluorescence analysis of HER2+ breast cancer biopsies revealed that CB2R-positive cancer cells presented nuclear ELK1 immunoreactivity (Figure R5C).

1.4. CB2R overexpression confers pro-oncogenic advantages on HER2+ breast cancer cells

We next analyzed the biological consequences of the HER2-induced CB2R upregulation. HER2 overexpression in MDA-MB-231 cells (which effectively enhanced CB2R levels, Supplementary Figure 4A) increased cancer cell viability (Figure R6A) and stimulated properties of cancer cells intimately related to tumor progression, *i.e.* invasion (Figure R6B) and anchorage-independent growth (Figure R6C). These effects were prevented by CB2R knock-down (Figure R6A-C). Likewise, genetic silencing of CB2R (Supplementary Figure 4B) reduced cell viability (Figure R6D), cell invasion (Figure R6E),

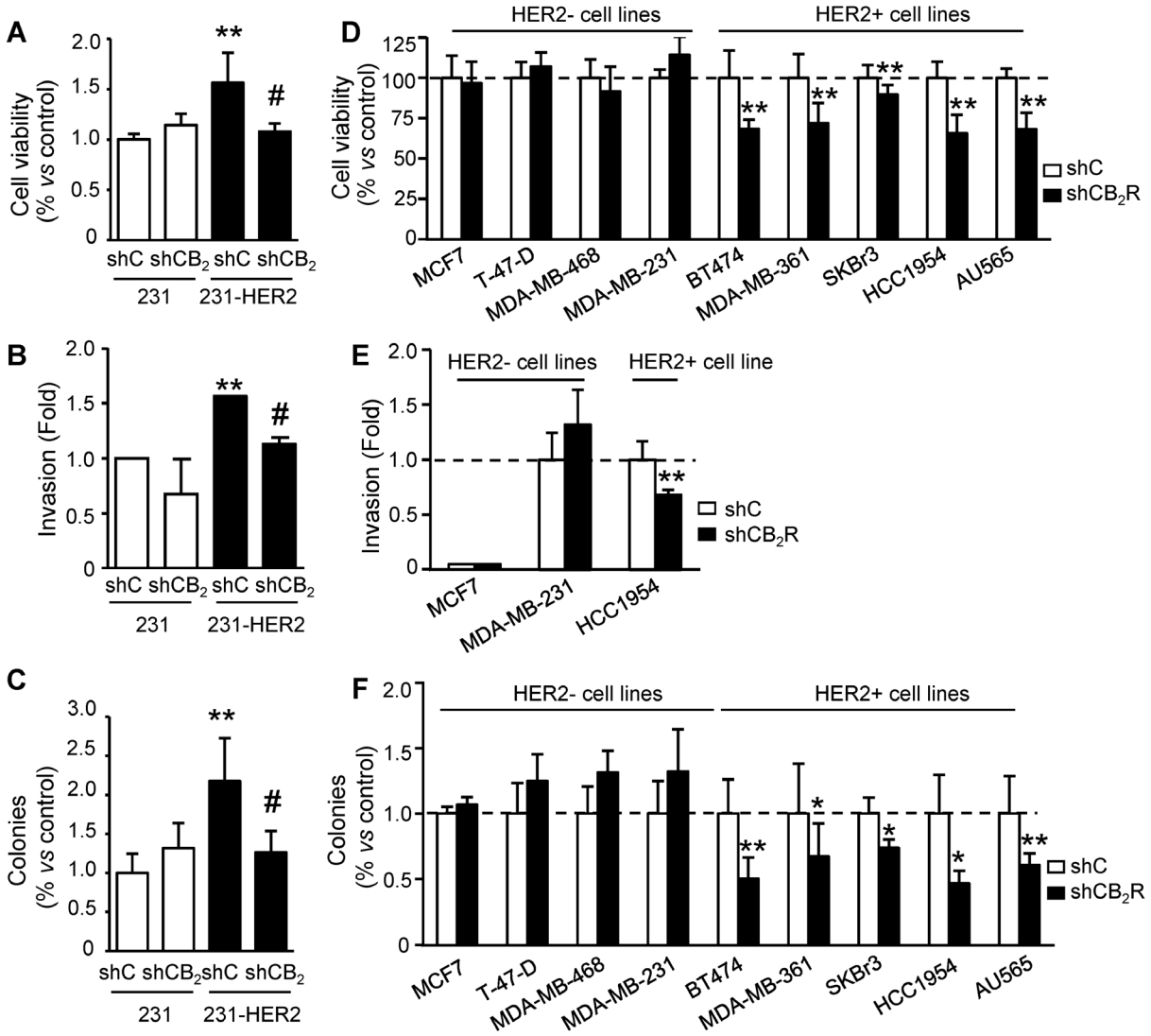


Figure R6. CB2R overexpression confers pro-oncogenic advantages on HER2+ breast cancer cells.
Cell viability as determined by the MTT test **(A, D)**, invasion in matrigel-coated Boyden chambers **(B, E)**, and number of colonies generated in soft agar **(C, F)** of MDA-MB-231 (231) and MDA-MB-231-HER2 cells (231-HER2) **(A-C)**, or of the indicated HER2-positive or HER2-negative cell lines **(D-F)** stably expressing a shRNA selectively targeting CB2R (shCB2R) or a scrambled shRNA (shC). Data were analyzed by ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test (n ≥ 3 independent experiments).*, p<0.05 and **, p<0.01 *vs* the corresponding shC cell line; #, p<0.01 *vs* shC-231-HER2 cells.

colony formation in soft agar (Figure R6F) and the expression of metastasis markers (Figure R7) in cells endogenously overexpressing HER2, an effect that was not observed in HER2-negative cells (Figure R6D-F).

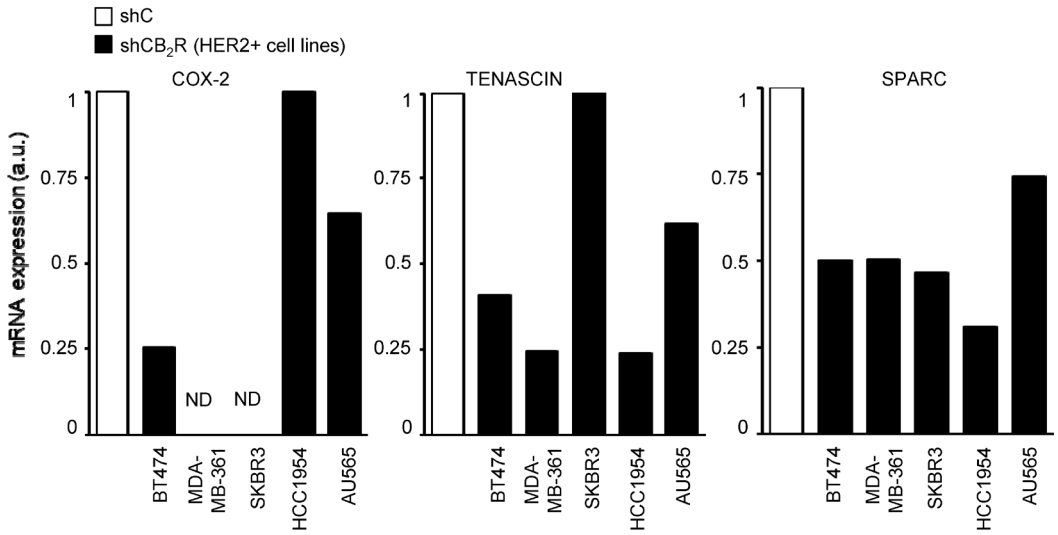


Figure R7.CB2R overexpression confers pro-oncogenic advantages on HER2+ breast cancer cells.
mRNA expression of the indicated proteins (as determined by Q-PCR) in HER2+ breast cancer cell lines after stable transfection with a shRNA selectively targeting CB2R (shCB2R) (black bars). Results are expressed in arbitrary units vs the corresponding shC transfected cells, set at 1 (white bars). ND, non detected.

To further investigate the *in vivo* relevance of our findings, we compared the effect of CB2R silencing on tumor growth of orthotopic xenografts bearing HER2- tumors (Figure R8A) *versus* animals bearing HER2+ tumors (Figure R8B). Interestingly, stable knock-down of CB2R exclusively reduced the growth of HER2 overexpressing tumors and not the HER2-negative tumors. Moreover, in a metastatis model generated by the injection of 231-HER2-LM cells (*i.e.* a lung-seeking metastatic variant of the MDA-MB-231-HER2 cell line) in the tail vein of mice, CB2R downregulation significantly reduced the formation of lung metastasis (Figure R8C-E). Similarly, tumors generated from cells that endogenously overexpress HER2 significantly reduced their growth when CB2R was silenced (Figure R8G-F). Collectively, these data show that CB2R promotes pro-oncogenic responses in a HER2+ context *in vitro* and *in vivo*.

1.5. Potential targets of CB2R-HER2 pro-oncogenic signaling

Next, we aimed at identifying CB2R targets responsible for its pro-tumoral activity. Upon modulation of CB2R and HER2 expression, and by means of a phospho-kinase array, we detected significant alterations in some members of the SRC family of non-receptor tyrosine kinases, which has been extensively related to cancer (Yeatman 2004; Kim *et al.* 2009; Zhang and Yu 2012). Specifically, we found that the expression of phosphorylated LYN, LCK, YES, FGR, HCK and FYN decreased upon CB2R knock-down (Figure R9A-B). However, this effect was observed both in HER2+ and HER2- cells (Figure R9A-B), which suggests that, although these may be relevant CB2R targets in breast cancer, they are not HER2+ context-specific. Of interest, the phosphorylated form of another member of the

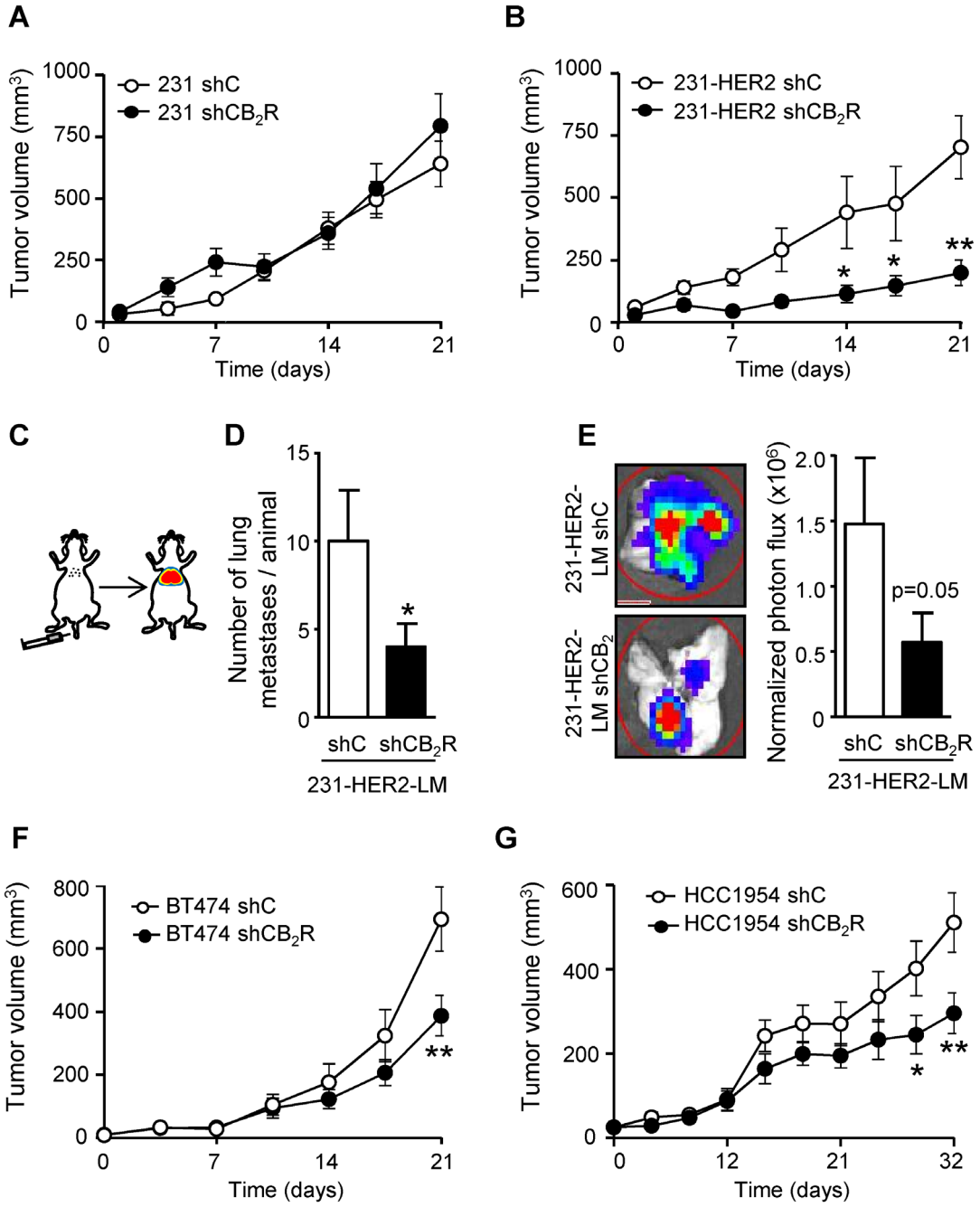


Figure R8. CB2R confers pro-oncogenic advantages on HER2+ breast cancer cells *in vivo.*
(A,B) Evolution of tumor volume in mice injected with either 231 **(A)** or 231-HER2 cells **(B)** stably expressing shCB2R or shC, or with the indicated HER2-amplified cells stably expressing the same shRNAs **(F,G)**. **(C)** Cartoon representing the generation of the lung metastases analyzed in **D** and **E** by injection of lung-seeking MDA-MB-231-HER2 cells (231-HER2-LM) stably expressing shCB2R, or a shC, into the mouse lateral tail vein. **(D)** Evaluation of the number of lung metastases generated per animal. **(E)** Representative lung bioluminescence images (left panels) and quantification of the luminescence signal (right panel) in the two experimental groups. Data were analyzed by ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test.*, $p<0.05$ and **, $p<0.01$ *vs* the corresponding shC cell line.

SRC family (c-SRC), which has particular relevance in cancer development and progression (Yeatman 2004), was specifically upregulated upon HER2 overexpression and downregulated by CB2R knock-down in that high-HER2 context (Figures R9B-C). Moreover, when CB2R expression was restored in MDA-MB-231-HER2 shCB2R cells, p-c-SRC levels were increased, an effect that was not evident in

the MDA-MB-231 that do not overexpress HER2 (Figure R9D). Likewise, a decrease in p-c-SRC upon CB2R silencing was observed in a panel of 5 breast cancer cell lines that endogenously overexpress HER2 (Figure R9E).

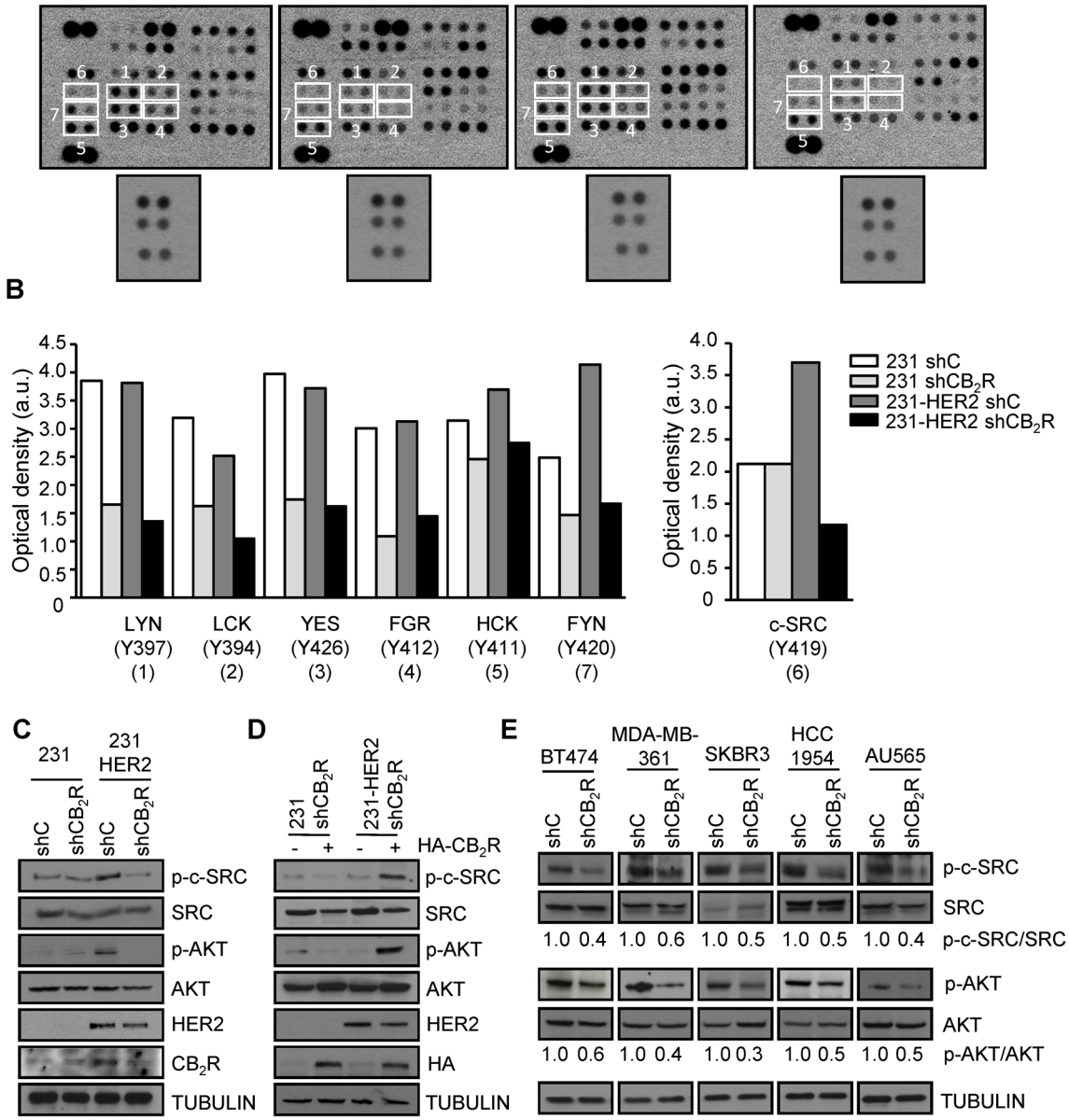


Figure R9. c-SRC as potential target of CB2R-HER2 pro-oncogenic signaling.
(A) Phospho-kinase protein array analysis in MDA-MB-231 (231) and MDA-MB-231-HER2 cells (231-HER2) after stable knockdown of CB2R by means of selective shRNA. Squared dots correspond to the following SRC kinase family members: 1, LYN; 2, LCK; 3, YES; 4, FGR; 5, HCK; 6, c-SRC and 7, FYN. Note that in this phosphoarray kit, each condition consist of two membranes. **(B)** Densitometric analysis of the squared dots shown in A. **(C-E)** Western blot analysis of the indicated proteins in MDA-MB-231 (231) and MDA-MB-231-HER2 (231-HER2) cells stably expressing a shRNA selectively targeting CB2R (shCB2R) or a scrambled shRNA (shC) **(C)**, or in 231 shCB2R or 231-HER2 shCB2R cells stably expressing a HA tagged-CB2R plasmid (HA-CB2R) or the corresponding empty vector (pcDNA3) **(D)**, or in the indicated HER2-amplified cells stably expressing shCB2R or shC **(E)**. Numbers in **C** correspond to the densitometric analysis of the respective bands.

1.6. Involvement of c-SRC in CB2R-induced HER2–mediated pro-oncogenic signaling

Next, we tested whether c-SRC was responsible for CB2R-driven oncogenesis. First, we observed that mouse NIH/3T3 embryonic fibroblasts acquire clonogenic properties upon overexpression of either CB2R or HER2 (Figure R10A-B). Moreover, the ability of these cells to form colonies in soft agar significantly increased when the two receptors were simultaneously overexpressed (Figure R10A-B). Disruption of c-SRC signaling by using a c-SRC dominant negative construct prevented the oncogenic phenotype induced by CB2R plus HER2 (Figure R10A-B). Of interest, while the HER2-mediated increased clonogenicity was prevented by blocking c-SRC signaling, the CB2R-induced clonogenic response was not (Figures R10A-B), which indicates that CB2R promotes c-SRC activation (and the subsequent clonogenic response) *via* HER2. We then performed colony formation experiments with human HER2-amplified breast cancer cells. Specifically, we overexpressed CB2R in 5 HER2+ cell lines and observed an increase in the levels of p-c-SRC (Figure R10C). As expected, this increase in activated c-SRC was accompanied by an enhanced clonogenicity (Figure R10D). Importantly, pharmacological inhibition of c-SRC with Saracatinib (a SRC family/Abl dual-kinase inhibitor) in CB2R-overexpressing cells kept both clonogenicity (Figure R10D) and p-c-SRC expression (Figure R10C) at the same level as in pcDNA3-transfected cells treated with the inhibitor, which further suggests that CB2R-driven oncogenesis is mediated by c-SRC activation.

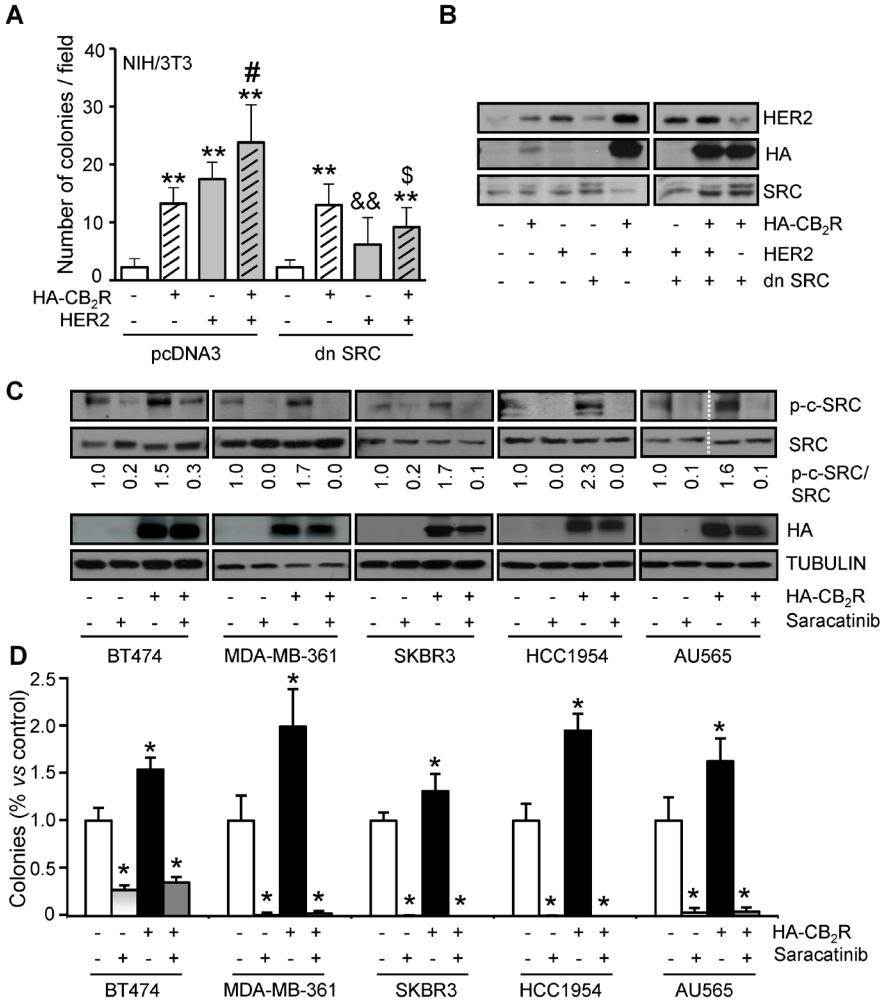


Figure R10. Involvement of c-SRC in CB2R-induced HER2-mediated pro-oncogenic signaling.
Anchorage-independent growth **(A, D)** and expression of HER2, HA-CB2R and the indicated proteins **(B, C)** (as determined by Western blot), in NIH/3T3 fibroblasts **(A, B)** and in a panel of 5 HER2+ human breast cancer cell lines **(C, D)** transiently transfected with the indicated constructs and/or incubated with 1μM Saracatinib. In **B** and **C** (AU565 cells), lanes were run on the same gel but were noncontiguous. Data were analyzed by ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test. *, p<0.05 and **, p<0.01 *vs* pcDNA3; #, p<0.05 *vs* CB2R or HER2; &&, p<0.01 *vs* HER2; $, p=0.05 *vs* HER2-CB2R test.*, p<0.05 and **, p<0.01 *vs* the corresponding shC cell line.

Finally, and in further support of a causal link between the CB2R/HER2/c-SRC axis and pro-oncogenic events, we found a decreased c-SRC and AKT activation in tumors generated by CB2R-deficient animals [which present a less aggressive phenotype (Figure R3)] with respect to their WT littermates (Figure 11A-B), and the analysis of human tumor biopsies revealed that HER2+ breast cancer cells expressing activated c-SRC also expressed CB2R (Figure R11C).

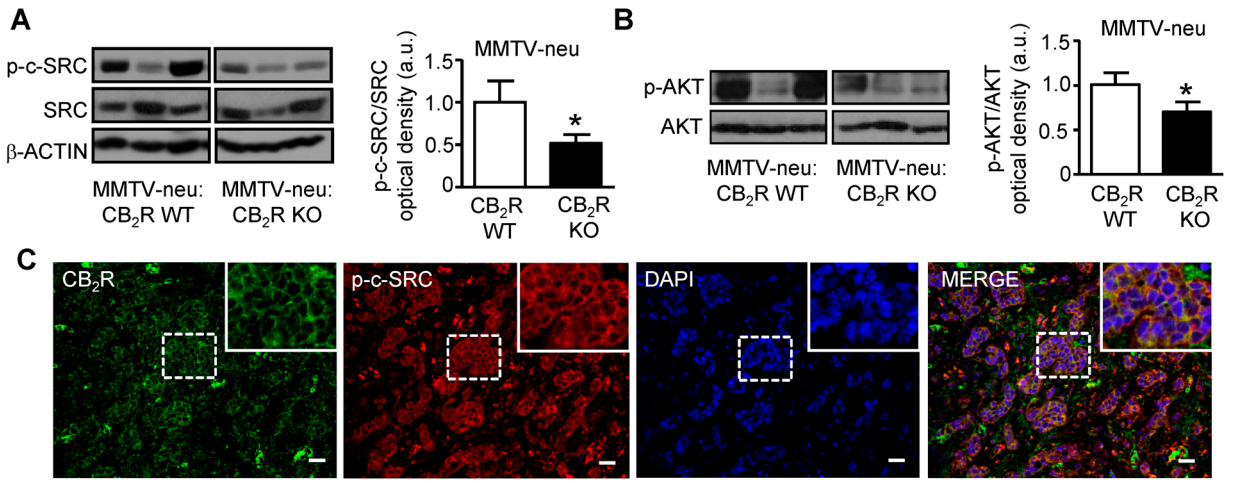


Figure R11. Involvement of c-SRC in CB2R-induced HER2-mediated pro-oncogenic signaling.
(A,B) Western blot (left panels) and densitometric analysis (right panels) of phospho-c-SRC **(A)** and phospho-AKT **(B)** in breast tumors generated by MMTV-neu: CB2R WT and MMTV-neu: CB2R KO mice. **(C)** Immunofluorescence analysis of CB2R (green) and phospho-c-SRC (red) in a human HER2+ breast tumor sample. Cell nuclei are stained in blue. Scale bar, 100μm. Data were analyzed by ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test (n=7 animals per group). *, $p<0.05$ *vs* MMTV-neu: CB2R WT mice **(A,B)**.

AIM2. ANALYSIS OF THE POTENTIAL PHYSICAL INTERACTION BETWEEN HER2 AND CB2R IN HER2+ BREAST TUMORS.

SUMMARY

As described in the previous section, CB2R expression is highly associated to HER2+ breast cancer and, in this context, higher levels of the cannabinoid receptor correlate with poor patient prognosis. In addition, we described that CB2R is an important component of the well-known HER2 pro-oncogenic machinery in breast cancer. On the basis of these findings, the second aim of this Thesis was to analyze whether the functional crosstalk between HER2 and CB2R was due to a molecular interaction between the receptors and, if so, to investigate the precise role of these novel complexes in breast cancer. To address this aim, we analyzed whether the putative HER2-CB2R heteromers fullfilled the three criteria required for demonstrating receptor heterodimerization (Gomes *et al.* 2016): (1) heteromer components should colocalize and physically interact; (2) heteromer components should exhibit properties distinct from those of the protomers, and (3) heteromer disruption should lead to a loss of heteromer-specific properties.

In this section of the Thesis we aimed at identifying and characterizing HER2-CB2R heteromers, which is intimately related to Criteria 1 and 2. Thus, we demonstrated the physical interaction between HER2 and CB2R by different techniques in breast cancer cells and in human breast cancer tissue. Moreover, we observed that the expression of these complexes correlates with poor patient prognosis. In addition, we found that CB2R couples to different heterotrimeric G proteins depending on whether it is part of the heteromer or not.

Altogether, our findings reveal the existence of a new heteromer between HER2 and a GPCR (CB2R) with prognostic value and potential as a new druggable therapeutic target in HER2+ breast cancer.

AIM2. ANALYSIS OF THE POTENTIAL PHYSICAL INTERACTION BETWEEN HER2 AND CB2R IN HER2+ BREAST TUMORS.

2.1. HER2 forms heteromers with CB2R

In the previous section of this Thesis we found a strong association between HER2 and CB2R in terms of expression and functionality. We therefore hypothesized that HER2 could be physically interacting with CB2R, forming heteromers. To test this hypothesis, we first performed co-immunoprecipitation assays. Immunoprecipitation of HER2 in HEK293 cells produced the co-precipitation of CB2R, and *vice versa* (Figure R12A). Additional evidence of heteromer formation was obtained by Biolumiscence Resonance Energy Transfer (BRET) experiments in HEK293 cells transiently transfected with HER2 fused to Rluc, and increasing concentrations of CB2R fused to YFP. The BRET signal increased as a saturation curve, indicating a specific interaction between both receptors. In contrast, a low and linear BRET was obtained for the negative controls (HER2-Rluc / GHS-R1a-YFP, and D44R-Rluc / CB2R-YFP) (Figure R12B-C). Further support for HER2-CB2R molecular interaction was obtained by Bimolecular Fluorescence Complementation (BiFC) assays. Briefly, constructs of HER2 and CB2R were fused at the C-terminal end to hemi-YFP Venus protein (nYFP and cYFP, respectively). If an interaction occurs, YFP reconstitution is achieved and manifested as fluorescent signal. This is precisely what we observed when both constructs were simultaneously transfected in HEK293 cells (Figure R12D-E). Results from these three different experimental approaches strongly suggest that there is a physical interaction between HER2 and CB2R in HEK293 transfected cells.

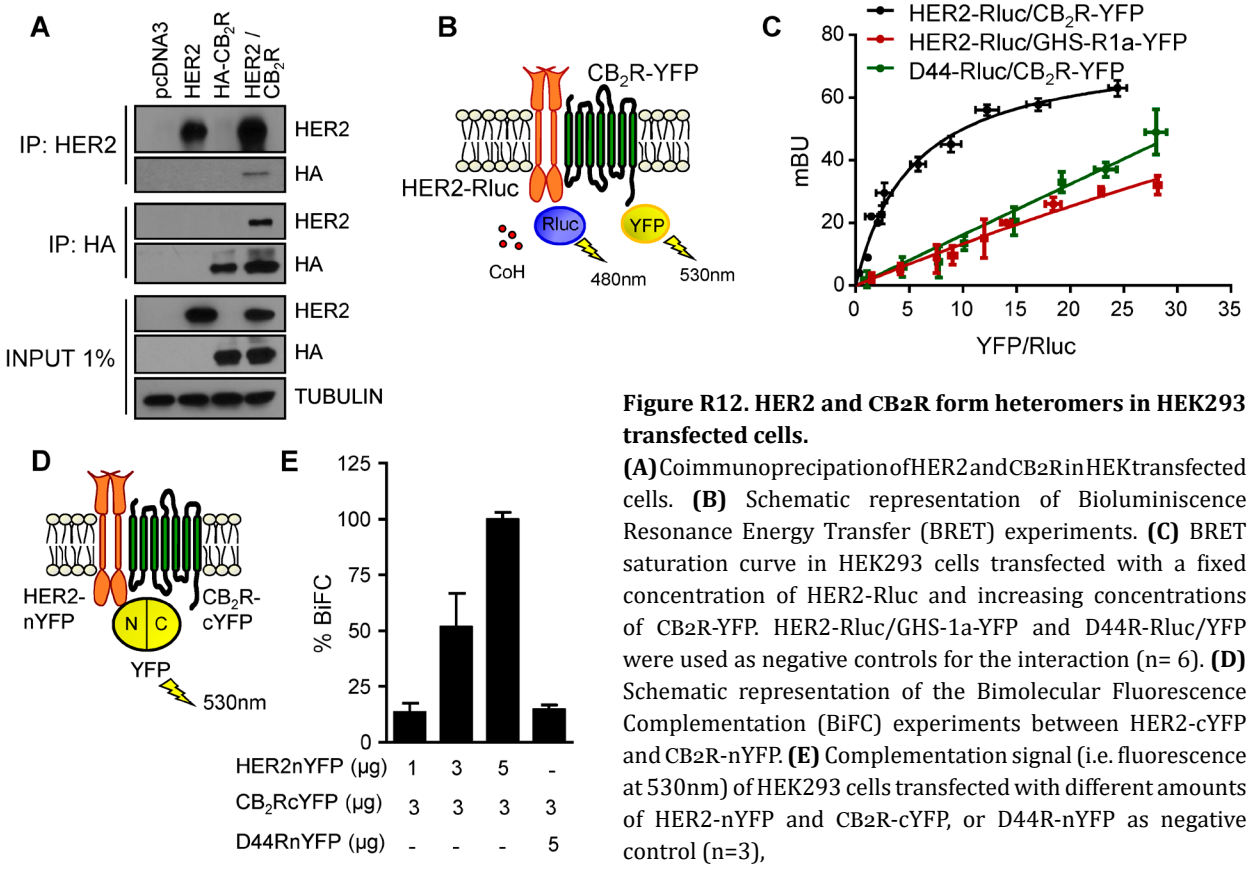


Figure R12. HER2 and CB2R form heteromers in HEK293 transfected cells.
(A) Coimmunoprecipation of HER2 and CB2R in HEK transfected cells. **(B)** Schematic representation of Bioluminiscence Resonance Energy Transfer (BRET) experiments. **(C)** BRET saturation curve in HEK293 cells transfected with a fixed concentration of HER2-Rluc and increasing concentrations of CB2R-YFP. HER2-Rluc/GHS-1a-YFP and D44R-Rluc/YFP were used as negative controls for the interaction (n= 6). **(D)** Schematic representation of the Bimolecular Fluorescence Complementation (BiFC) experiments between HER2-cYFP and CB2R-nYFP. **(E)** Complementation signal (i.e. fluorescence at 530nm) of HEK293 cells transfected with different amounts of HER2-nYFP and CB2R-cYFP, or D44R-nYFP as negative control (n=3),

To determine whether these complexes were also present in more physiological settings, we performed a series of experiments in native conditions, without overexpression of any of the two receptors. Specifically, we performed proximity ligation assays (PLA) (Figure R13A) (Fredriksson *et al.* 2002). The presence of HER2-CB2R heterodimers was detected as red fluorescent dots in human breast cancer cells that endogenously overexpress HER2 and CB2R (Figure R13B), and this fluorescent signal was not evident when CB2R was stably silenced (Figure R13B). These results strongly suggest that HER2 and CB2R form heteromers in breast cancer cell lines.

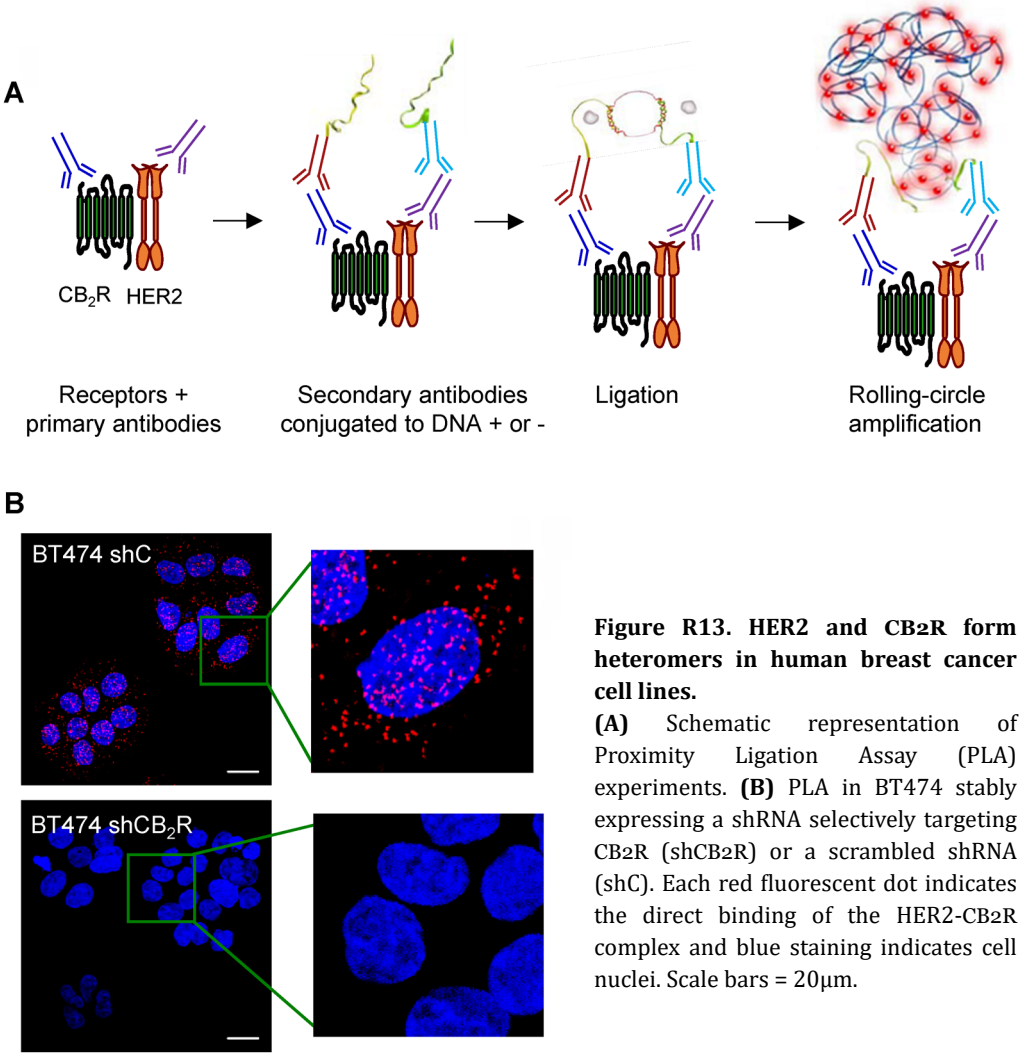


Figure R13. HER2 and CB2R form heteromers in human breast cancer cell lines.
(A) Schematic representation of Proximity Ligation Assay (PLA) experiments. **(B)** PLA in BT474 stably expressing a shRNA selectively targeting CB2R (shCB2R) or a scrambled shRNA (shC). Each red fluorescent dot indicates the direct binding of the HER2-CB2R complex and blue staining indicates cell nuclei. Scale bars = 20μm.

We next tried to confirm the presence of these heteromers in human breast cancer tissue. First, the immunofluorescence analysis of human HER2+ breast cancer samples revealed that the two receptors colocalize (Figure R14A). Moreover, PLAs confirmed the presence of HER2-CB2R heteromers in HER2+ tumors but not in HER2- samples (Figure R14B-C). Importantly, CB2R expression was much higher in HER2+ tumors than in HER2- tumors as it has been previously reported in the first aim of this Thesis (Figure R14B). The specificity of the HER2-CB2R PLA signal was confirmed in tumor

samples from MMTV-neu mice: *i.e.*, this signal was absent in the CB2R knock-out animals and positive in their wild type littermates (Figure R14D-E). Altogether, these results demonstrate the physical interaction between HER2 and CB2R in native breast cancer tissue.

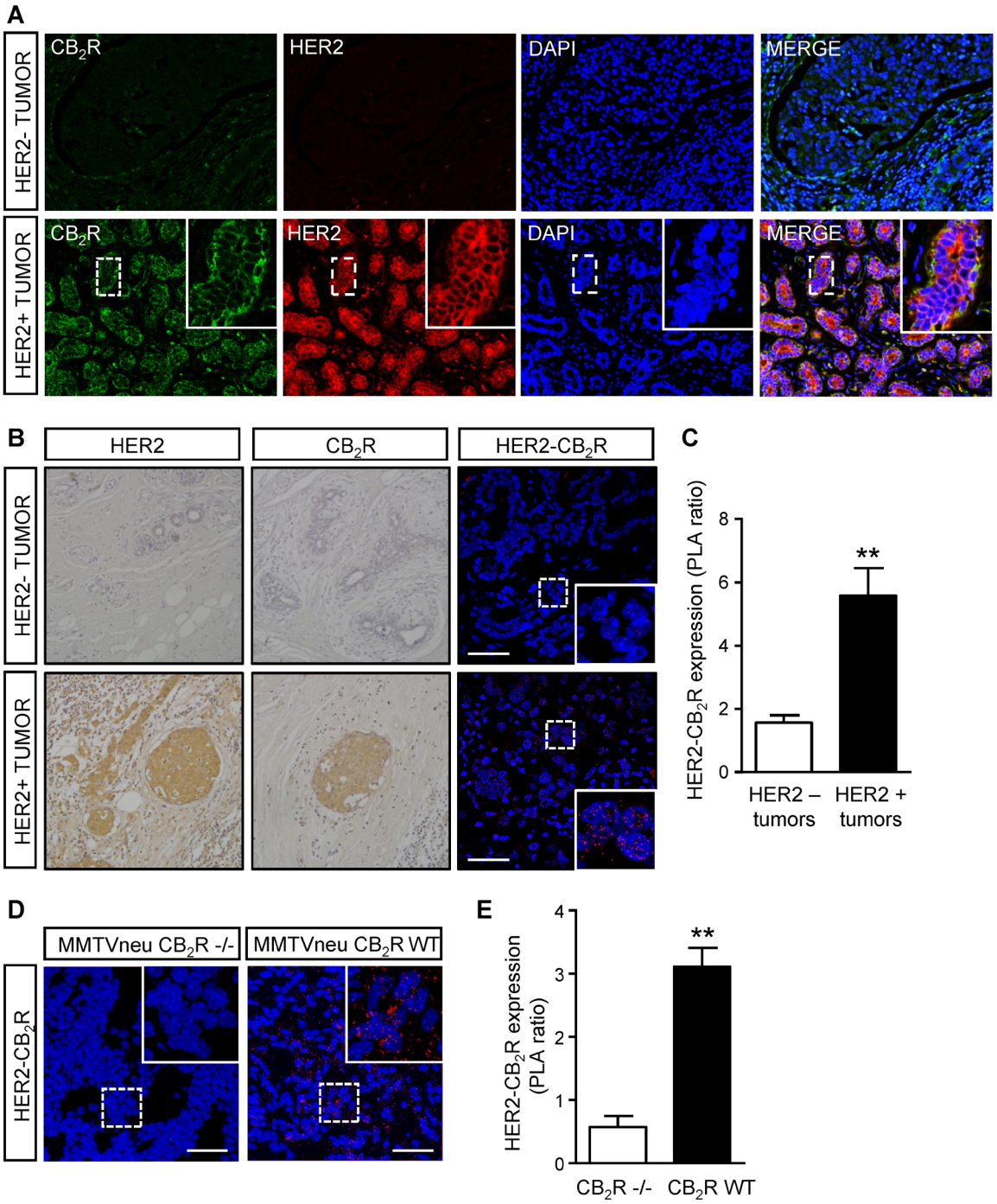


Figure R14. HER2 and CB2R form heteromers in human breast cancer tissue.
(A) Immunofluorescence analysis of CB2R (green) and HER2 (red) protein expression in HER2-negative (upper panels) and HER2-positive (lower panels) human breast tumor samples. Cell nuclei are stained in blue. Scale bar = 100μm **(B)** Representative images of HER2 and CB2R individual expression by IHC (left and central panels), and HER2-CB2R heteromer expression by PLA (right panels) in human samples from HER2 – (upper panels) and HER+ (lower panels) tumors. **(C)** Quantification of the corresponding HER2-CB2R heteromer signal (in red), represented as number of red dots per cell (cell nuceli in blue), in 4-9 different fields per sample. **(D)** Representative images of HER2-CB2R heteromer expression in tumor samples from MMTVneu: $CB2R^{-/-}$ or CB2RWT animals, and its corresponding quantification (n=3 per genotype) **(E)**. Scale bars: 50μm. Data are represented as mean ± SEM of counts in 4–9 different fields. Unpaired, 2-tailed Student's t test: ** $p < 0.01$ *vs* HER2- tumors **(C)** or $CB2R^{-/-}$ animals **(E)**.

2.2. HER2-CB2R heteromer expression correlates with poor patient prognosis

To determine whether the expression of these newly identified complexes correlates with any clinically relevant information, we analyzed the expression of HER2-CB2R heteromers in a series of 57 human HER2+ breast cancer biopsies obtained at the time of first diagnosis, before neoadjuvant treatment (TMA #3 in the Methods sections). PLAs (Figure R15A) showed that higher HER2-CB2R expression in the tumors is associated to lower disease-free patient survival (Figure R15B), as well as to higher spread to regional lymph nodes and Ki67 overexpression (Table R1). To further validate these observations, we performed similar analyses in an additional TMA containing 39 human high-grade HER2+ ductal breast cancer samples obtained before any treatment (TMA #4 in the Methods section). High HER2-CB2R heteromer expression was also associated to poor patient prognosis, specifically lower disease-free (Figure R15C) and overall patient survival (Figure R1D).

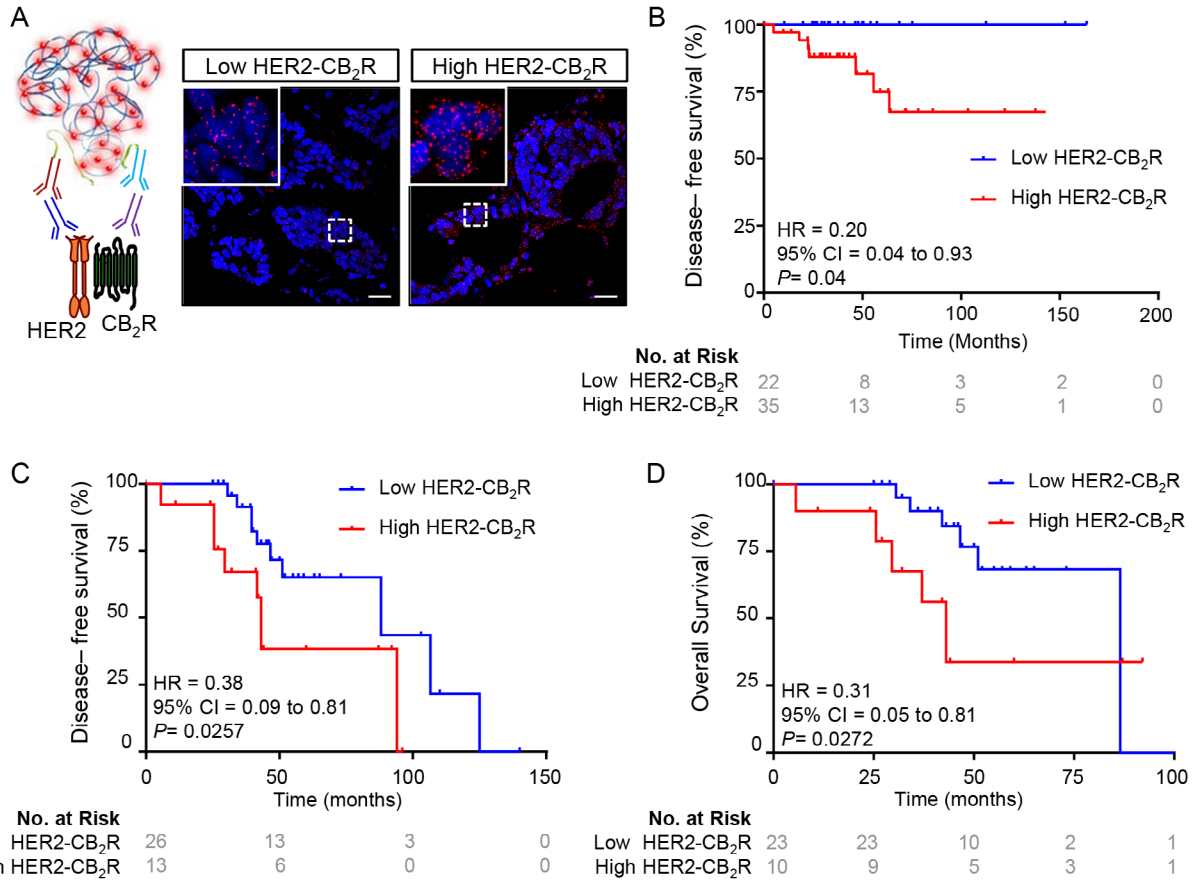


Figure R15. HER2-CB2R heteromer expression correlates with poor patient prognosis.
Proximity Ligation Assays (PLAs) were performed in Tissue Microarrays (TMAs #3 and #4). Samples were ranked based on HER2-CB2R heteromer expression (*i.e.* PLA signal), and the best cut-off was manually selected. **(A)** Representative confocal microscopy images of a low- and a high-heteromer expressing sample. The red dotted signal corresponds to the heteromers, and the blue staining to cell nuclei. Scale bars, 25µm. **(B-D)** Kaplan-Meier curves for disease-free survival [from samples included in TMA #3] **(B)**, and disease-free **(C)** and overall patient survival **(D)** (from samples included in TMA #4). Curves were statistically compared by the log-rank test (* p <0.05).

Table R1. Clinical/pathological features of the tumor samples included in TMA #3.
The Student´s t test was used to analyze associations between categorical variables. All tests were 2-tailed.

Factors	*Sample size (% of total)*	*PLA ratio*	*p Value*	
Age (years)				
<50	29 (51%)	7,6 ± 2,7	0,258	
≥50	28 (49%)	7,0 ± 2,4		
T stage				
T1-T2	29 (47%)	7,8 ± 2,6	0,235	T1-T2 *vs* T3
T3	8 (13%)	6,8 ± 2,0	0,379	T3 *vs* T4
T4	24 (40%)	6,4 ± 2,4	0,083	T1-T2 vs T4
N stage				
N0	20 (36%)	6,8 ± 1,3	0,448	N0 *vs* N1
N1	28 (51%)	6,7 ± 3,1	0,221	N1 *vs* N2N3
N2-N3	7 (13%)	8,1 ± 1,7	**0,050**	N0 *vs* N2N3
Grade				
II	21 (49%)	7,1 ± 2,8	0,322	
III	22 (51%)	7,6 ± 2,2		
ER status				
Negative (<10%)	20 (42%)	7,4 ± 2,2	0,269	
Positive (>10%)	28 (58%)	7,0 ± 2,3		
PR status				
Negative	30 (56%)	7,5 ± 2,8	0,348	
Positive	24 (44%)	7,1 ± 2,3		
Ki67 status				
Normal (<30%)	20 (38%)	5,9 ± 2,3	**0,005**	
Overexpression (>30%)	32 (62%)	8,4 ± 2,4		
p53 status				
Normal (<15%)	28 (53%)	6,9 ± 2,3	0,162	
Overexpression (>15%)	25 (47%)	7,8 ± 2,9		
Menopausal status				
Pre-menopausal	32 (56%)	7,5 ± 2,7	0,234	
Post-menopausal	25 (44%)	6,9 ± 2,4		
Subtype status				
Luminal B	34 (63%)	7,1 ± 2,5	0,251	
HER2	20 (37%)	7,8 ± 2,6		

The separate analysis of either HER2 or CB2R expression by immunohistochemistry confirmed that increased heteromer expression is not just a consequence of individual receptor overexpression. Thus, similar HER2-CB2R heteromer levels were found in tumors with low, medium or high HER2 expression (Figure R16A), as well as with no, low, medium or high CB2R expression (Figure R16B).

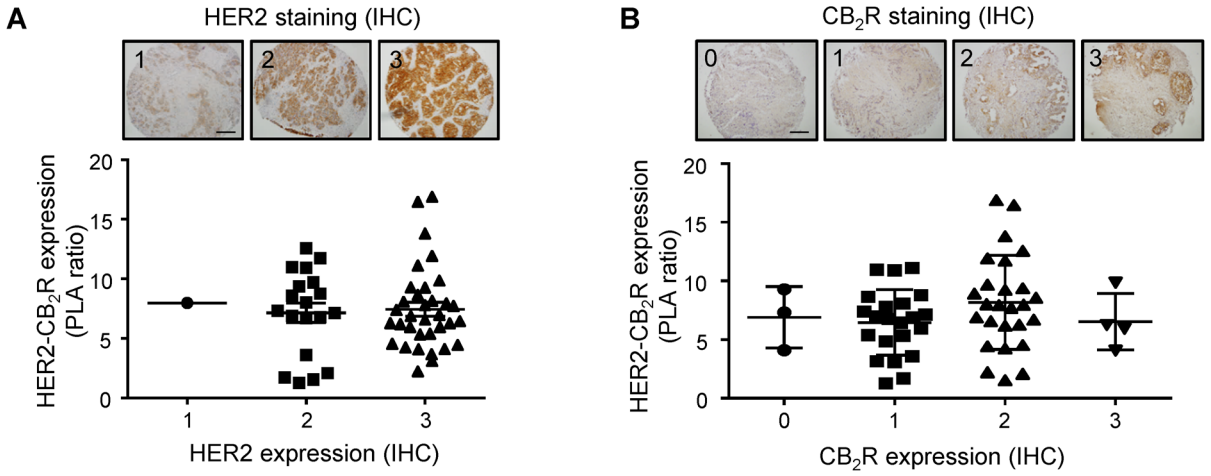


Figure R16. (A, B) Upper panels, representative images of HER2 **(A)** and CB2R expression **(B)**, as determined by immunohistochemistry (IHC) in the TMA samples. For HER2 expression, samples were scored according to HercepTest manufacturer's instructions. For CB2R expression, they were scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (high staining). Scale bar: 200µm. Lower panels, HER2-CB2R heteromer expression plotted against HER2 **(A)** or CB2R **(B)** expression. No statistically significant associations were found (1-way ANOVA).

Finally, we analyzed heteromer expression in two pairs of patient-derived xenografts (PDX). Each pair consisted of one PDX generated from the patient's primary tumor, and another PDX generated from the corresponding metastasis (in the liver in one case, and in a lymph node in the other). Consistent with the idea that HER2-CB2R complexes correlate with poor patient prognosis, in both

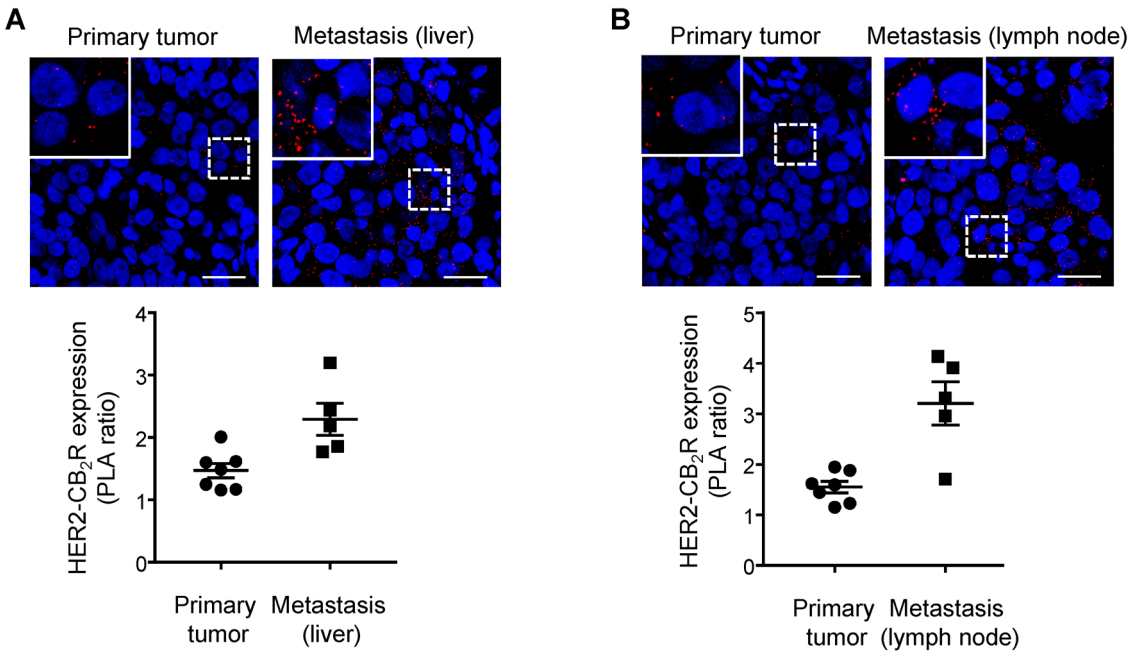


Figure R17. HER2-CB2R heteromer expression is increased in metastatic tissue compared to primary tumor. Proximity Ligation Assays (PLAs) were performed in patient-derived xenografts (PDX). **(A,B)** Upper panels, representative images of HER2-CB2R heteromer expression in two pairs of PDX, consisting on a PDX established from the patient's primary tumor and from a metastasis of the same patient [in the liver in one case, **(A)**, and in a lymph node in the other **(B)**]. Lower panels, quantification of HER2-CB2R heteromer expression in the PDX samples. Results are expressed as PLA ratio (number of red dots per cell, n=5-7 fields).

cases, we observed significantly higher heteromer expression in the metastatic tissue with respect to the corresponding primary tumor (Figures R17A-B). Together, these results demonstrate that HER2-CB2R heteromers are specific receptor complexes present in HER2+ breast cancer tissue that are associated to tumor recurrence and spreading.

2.3. The HER2-CB2R heteromer exhibits a specific signaling fingerprint

An essential requirement for receptor complexes to be considered functional heteromers is that they have to signal differently than the individual protomers. To determine whether this is the case for HER2-CB2R complexes, we analyzed the coupling of CB2R to different effector proteins in the presence or in the absence of HER2, and upon THC activation. First, we studied β-arrestin recruitment by BRET in HEK cells transfected with β-arrestin-Rluc, CB2R-YFP and an untagged HER2. We found that THC induced the interaction of CB2R with β-arrestin II (and not β-arrestin I). However, this recruitment was not affected by HER2 co-expression (Figure R18A-B). Next, we analyzed CB2R coupling to a variety of heterotrimeric G proteins after THC exposure. Interestingly, we found that the cannabinoid receptor couples to different G proteins depending on whether it is part of the heteromer or not (Figure R18C). Thus, in cells expressing just CB2R, THC induced the coupling of the receptor to $G_{q/11}$, while it promoted the coupling to G_i and G_z when HER2 and CB2R were coexpressed (Figure R18C). These findings suggest that the heteromer exhibits a specific signaling fingerprint, and support the idea that HER2-CB2R heteromers are unique signaling complexes.

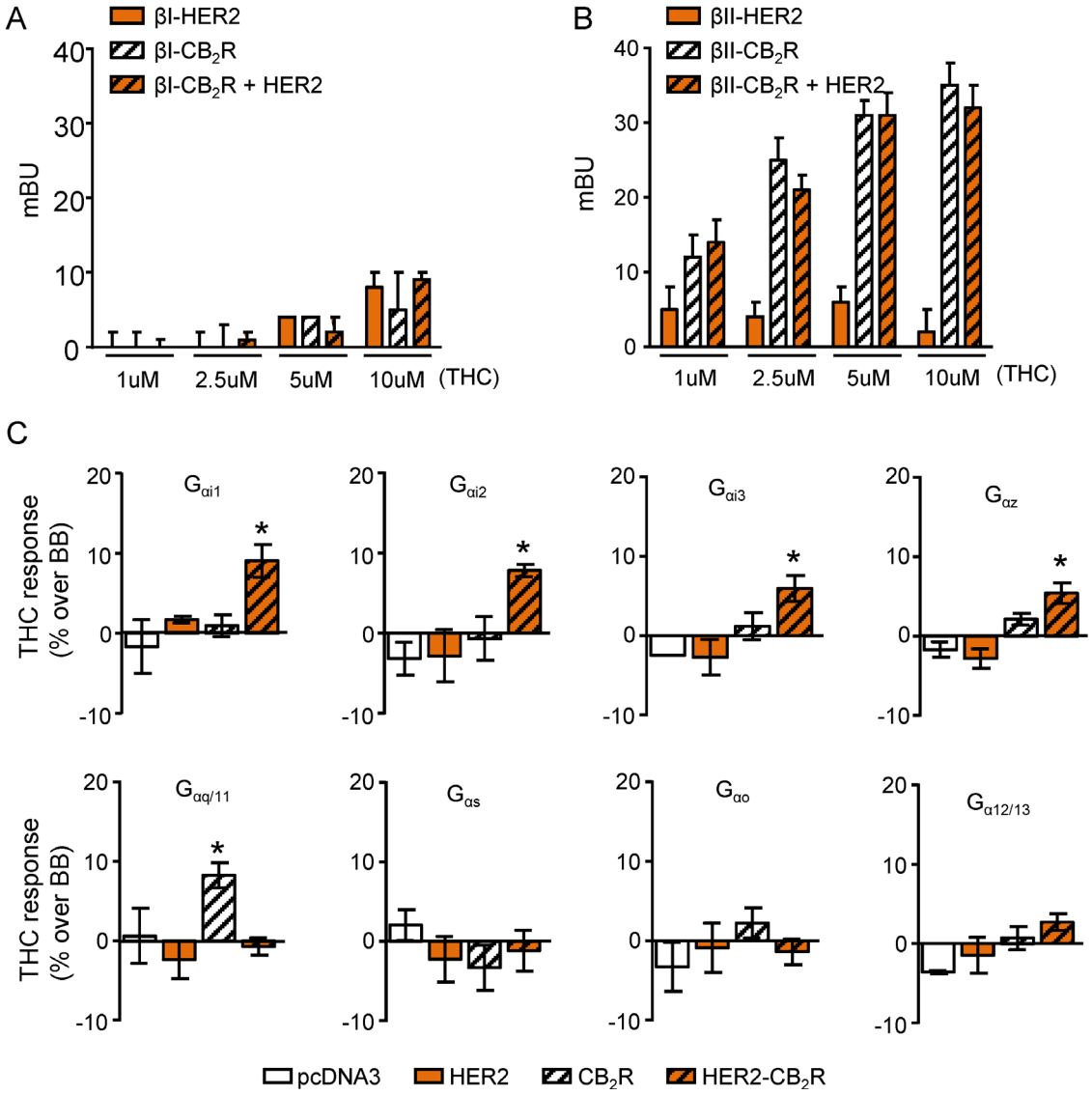


Figure R18. Characterization of the HER2-CB2R heteromer signaling.
(A-B) β-Arrestin recruitment was measured by Bioluminiscence Resonance Energy Transfer (BRET). HEK cells transtienly overexpressing HER2-YFP, or CB2R-YFP, or both receptors (CB2R-YFP + HER2-untagged) were co-transfected with either β-Arrestin I (βI) **(A)** or β-Arrestin II (βII) **(B)**. BRET signal (*i.e.* mBU) was measured after 20min treatment with THC at the indicated concentrations. Data from a minimum of 3 experiments are expressed as THC response (*i.e.* mBU) *vs* vehicle-treated cells, set at 0. **(C)** Activation of the indicated G proteins by THC, as determined by Antibody-capture [^{35}S]GTPγS scintillation proximity assay (SPA), in HEK293 cells transiently overexpressing HER2, CB2R, both receptors (HER2-CB2R) simultaneously or the corresponding empty vector (pcDNA3). Results are expressed as percentage of [^{35}S]GTPγS basal binding (BB, binding obtained in the absence of the agonist, set as 100% for each cell line). Data from a minimum of 3 experiments are expressed as % *vs* vehicle-treated cells, set at 100%. Multigroup comparisons were analyzed by One-way ANOVA with Tukey's post hoc test. *, p <0.05; **, p <0.01 *vs* vehicle-treated cells.

AIM3. ANALYSIS OF HER2-CB2R HETEROMERS AS POTENTIAL THERAPEUTIC TARGETS IN HER2+ BREAST CANCER

SUMMARY

Although the use of HER2-targeted therapies has dramatically improved the clinical outcome of HER2-positive breast cancer patients, a non-small percentage of them present either innate or acquired resistance to these treatments, and eventually progress. New therapies are therefore warranted for this patient sub-population, as well as new tools to early identify those at a higher risk of recurrence and progression.

Cannabinoids produce antitumor responses in preclinical models of cancer, including of HER2+ breast cancer (Caffarel *et al.* 2012; Schwarz *et al.* 2018; Velasco *et al.* 2012; Blasco-Benito *et al.* 2018). In most cases, the antitumor responses are elicited by binding and activation of cannabinoid receptors. In the previous sections of this Thesis we described the existence of a novel heteromer formed by HER2 and CB2R, which is associated to pro-oncogenic features. In this context, our third aim was to determine whether these novel structures are involved in cannabinoid antitumor action, and, in general, whether these heteromers may be new druggable targets for the management of HER2+ breast cancer.

First, we identified the CB2R region involved in the physical interaction between HER2 and CB2R. Specifically, we found that the transmembrane domain 5 of CB2R is crucial for HER2-CB2R heterodimerization. In addition, we demonstrated that disruption of HER2-CB2R complexes, which was achieved either by pharmacological manipulation or sterical competition, triggered antitumor responses by preventing the activation of HER2 and promoting its proteasomal degradation by the E3 ligase c-CBL.

Together, these observations not only shed light on the CB2R region physically involved in HER2-CB2R heteromer formation, but also suggest that disruption of these complexes may be a new therapeutic approach to fight against HER2+ breast cancer.

AIM3. ANALYSIS OF HER2-CB2R HETEROMERS AS POTENTIAL THERAPEUTIC TARGETS IN HER2+ BREAST CANCER

3.1. Disruption of HER2-CB2R complexes by THC impairs HER2+ breast cancer cell viability

As HER2-CB2R heteromer expression seems to be linked to pro-oncogenic processes, we studied whether these complexes could be targets for antitumor therapies. It has been previously described that CB2R activation leads to cancer cell death by apoptosis, and inhibition of tumor growth, angiogenesis and metastasis in different models of HER2+ breast cancer (Caffarel *et al.* 2010; Nasser *et al.* 2011). To determine if HER2-CB2R heteromers are involved in this cannabinoid antitumor action, we analyzed their expression in response to THC (the main bioactive constituent of cannabis). We first used HEK293 cells transiently transfected with HER2 and CB2R, and confirmed that THC induces a decrease in cell viability in this cell model (Figure R19A). By performing BRET, we found a significant reduction in the heteromer signal upon cannabinoid treatment when using a THC concentration that significantly reduced cell viability (Figures R19C-D). The cannabinoid-induced decrease in both HER2-CB2R and cell viability relied on CB2R activation, as pointed by the preventive effect of the CB2R-selective antagonist SR144528 (SR2) (Figure R19B and D).

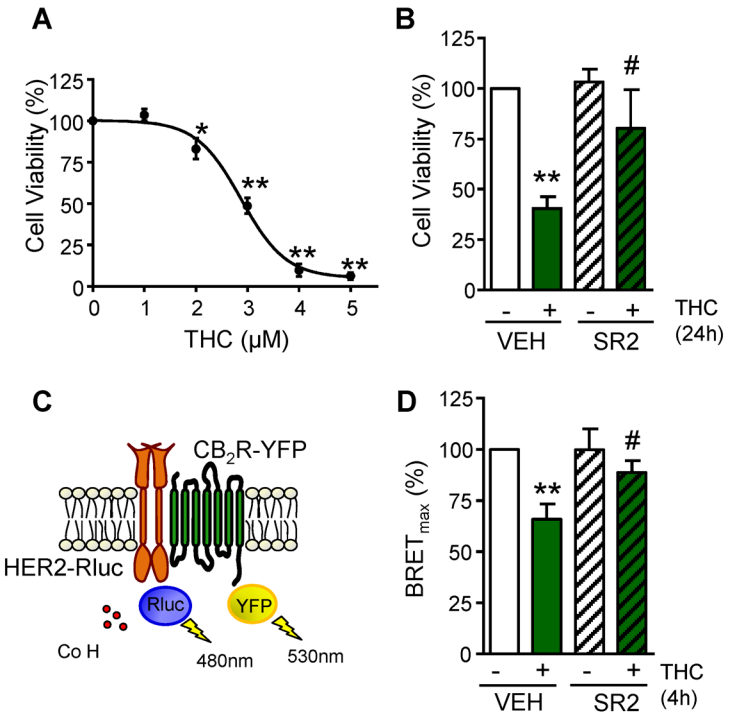


Figure R19. THC decreases HER2-CB2R complexes in HEK293 cells.
(A-B) Viability of HEK293 cells after 24h treatment with increasing concentrations of THC **(A)**, or THC in combination with the CB2R-selective antagonist SR2 (1μM) **(B)**. **(C)** Schematic representation of Bioluminiscence Resonance Energy Transfer (BRET) experiments. **(B)** Effect of THC (4h), alone or in combination with SR2 (1μM), on HER2-Rluc/CB2R-YFP BRETmax signal in HEK293 cells. Results (n=3-6 independent experiments) are expressed as % *vs* vehicle-treated cells, set at 100. Multigroup comparisons were analyzed by One-way ANOVA with Tukey's post hoc test. *, p <0.05; **, p <0.01 *vs* vehicle treated cells; #, p <0.05; ##, p <0.01 *vs* THC.

To determine whether the effects observed in HEK293 cells also occur in more physiological settings, we run a series of experiments in two different human HER2+ breast cancer cell lines (BT474 and HCC1954). THC decreased the viability in both cell lines in a concentration-dependent manner (Figure R20A), an effect that was again prevented by CB2R antagonism (Figure R20B). The

presence of HER2-CB2R heteromers in these cells was then analyzed by co-immunoprecipitation upon overexpression of a tagged form of CB2R. THC treatment diminished the amount of CB2R that co-immunoprecipitated with HER2 in both cell lines, which points to a cannabinoid-induced disruption of the heteromer (Figure R20C). To further support this idea, we performed PLAs in the two breast cancer cell lines in native conditions [*i.e.* under no overexpression of HER2 or CB2R)]. Data showed that THC decreases the amount of heteromers by activating CB2R (Figure R20D-E).

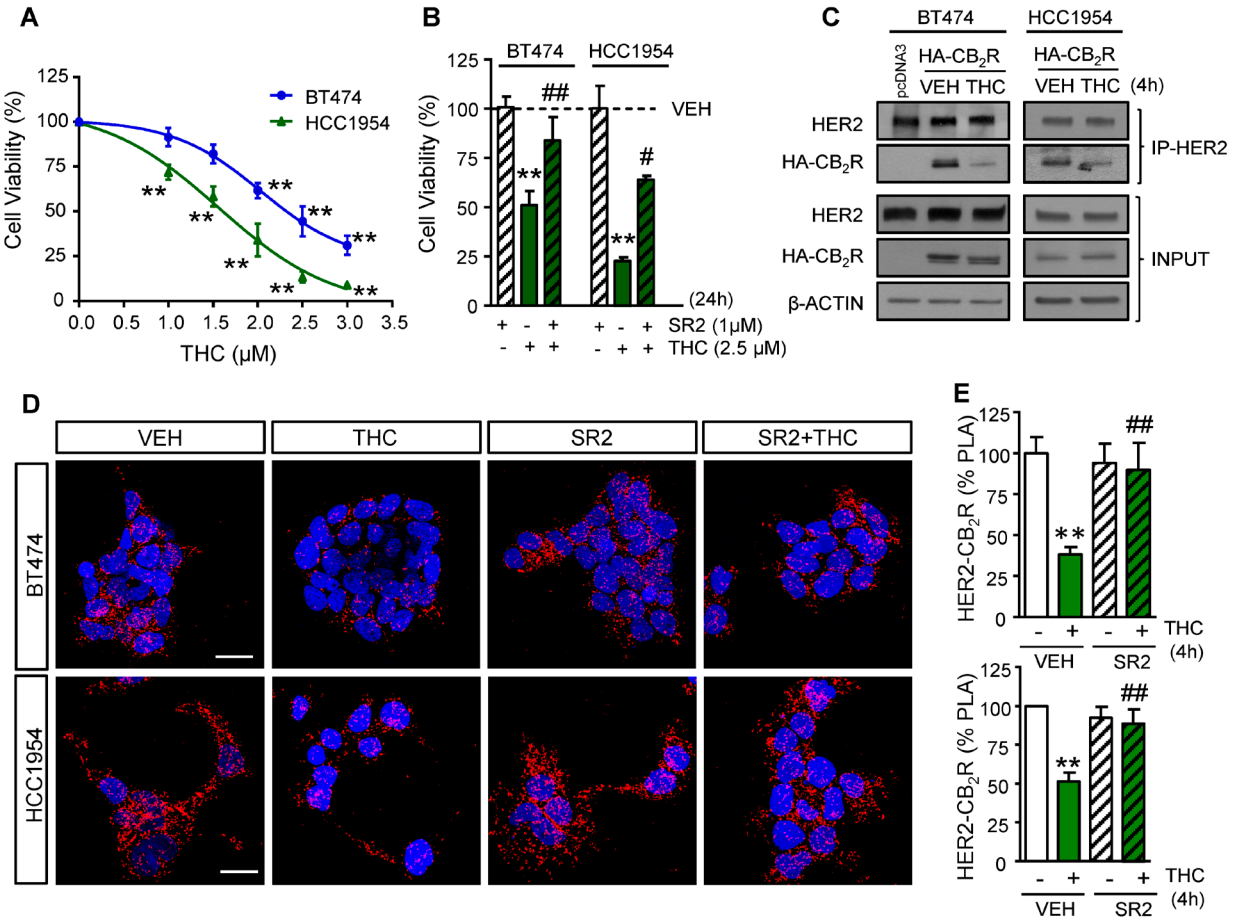


Figure R20. THC decreases HER2-CB2R complexes in HER2+ breast cancer cells
(A,B) Viability of BT474 and HCC1954 cells in response to increasing concentrations of THC **(A)**, or in combination with the CB2R-selective antagonist SR144528 (SR2, 1μM) **(B)**. Results (n=3-6 independent experiments) are expressed as % *vs* vehicle-treated cells, set at 100%. **(C)** Co-immunoprecipitacion of HER2 with CB2R after THC treatment (4h), in BT474 and HCC1954 cells transfected with a HA-tagged CB2R plasmid. **(D)** Representative PLA confocal microscopy images of HER2-CB2R heteromers (in red) in BT474 (upper panels) and HCC1954 cells (lower panels), treated with THC (4h) alone or in combination with SR2 (1μM). Cell nuclei are stained in blue. Scale bars: 25μm. **(E)** Quantification of HER2-CB2R PLA signal (number of red dots per cell), in a minimum of 3 independent experiments. Results are expressed as % *vs* vehicle-treated cells, set at 100%. One-way ANOVA with Tukey's post hoc test. *, p <0.05; **, p <0.01 *vs* vehicle-treated cells; #, p <0.05; ##, p <0.01 *vs* THC.

3.2. HER2-CB2R heteromer disruption by THC hampers HER2 activation

HER2 activation occurs upon dimerization with other members of the HER family, followed by trans and autophosphorylation of the intracellular domains of each protomer (Lemmon and Schlessinger 2010). We analyzed whether disruption of the HER2-CB2R heteromer had any effect

on this activation process. First, and to determine which specific HER dimers may be affected by HER2-CB2R disruption, we evaluated the expression of the four members of the HER family in the two HER2+ cell lines used in our studies. We found HER1 and HER3 overexpression in at least one of them when compared to a luminal (MCF7) or a basal (MDA-MB-231) breast cancer cell line (Figure R21A). We therefore studied the effect of THC on HER2-HER1, HER2-HER2 and HER2-HER3

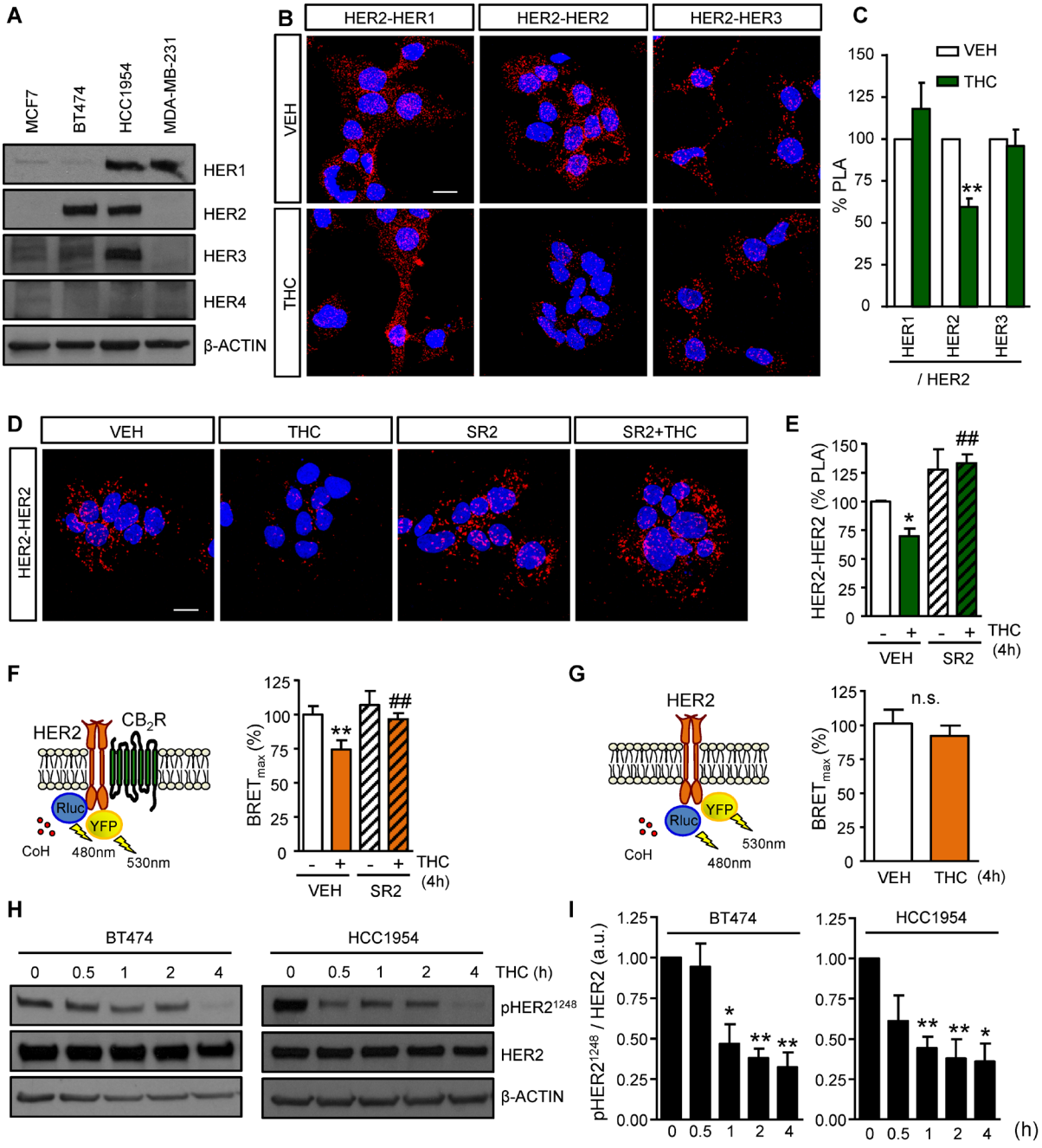


Figure R21. THC decreases HER2-HER2 homodimers.
(A) HER1, HER2, HER3 and HER4 expression, as determined by Western blot analysis, in the indicated breast cancer cell lines. **(B)** Representative PLA confocal miscroscopy images of the effect of THC (4h) on HER2-HER1, HER2-HER2 and HER2-HER3 dimers (in red) in HCC1954 cells **(B),** with the corresponding quantification **(C)**, or on HER2-HER2 expression after THC treatment, alone or in combination with the CB2R-selective antagonist SR144528 (SR2, 1μM) **(D)**, with the corresponding quantification **(E)**. Cell nuclei are in blue. Scale bars: 20μm. Results are expressed as % *vs* vehicle-treated cells, set at 100%. Multigroup comparisons were analyzed by One-way ANOVA with Tukey's post hoc test. *, p <0.05; **, p <0.01 *vs* vehicle-treated cells; ##, p <0.01 *vs* THC.

heteromers in HCC1954 cells. Neither HER2-HER1 nor HER2-HER3 complexes were diminished upon cannabinoid treatment (Figure R21B-C). In contrast, THC significantly diminished the amount of HER2-HER2 homodimers (Figure R21B-C), and this effect was prevented by SR2 (Figure R21D-E). HER2-HER2 homodimer reduction upon THC challenge, and involvement of CB2R in this effect, were further confirmed by BRET in HEK293 HER2-CB2R overexpressing cells (Figure R21F). As expected, THC produced no such effect in HEK293 cells lacking CB2 receptors (Figure R21G). In line with these observations, THC decreased the levels of HER2 phosphorylated in Tyr1248 (Figure R21H-I), one of the main autophosphorylation sites in this receptor. In summary, these observations demonstrate that HER2-CB2R heteromer disruption by THC hampers HER2 activation by interfering with its homodimerization.

3.3. THC induces HER2-CB2R heteromer disruption and HER2 degradation *in vitro* and *in vivo*

Our results show that THC inhibits HER2 pro-oncogenic signaling not only by hampering HER2 activation but also by promoting its degradation. Thus, cannabinoid challenge produced a marked decrease in the levels of activated (phospho-Tyr1248) HER2 (Figure R22A-B), that was followed by a decrease in the total levels of HER2, that was evident after 6-8 hours of treatment, depending on the specific cell line (Figures R22A-B). This effect was prevented by blockade of CB2R (Figure R22C) and was not due to inhibition of gene transcription, as indicated by the observation that HER2 mRNA levels remained unchanged (Figure R22D).

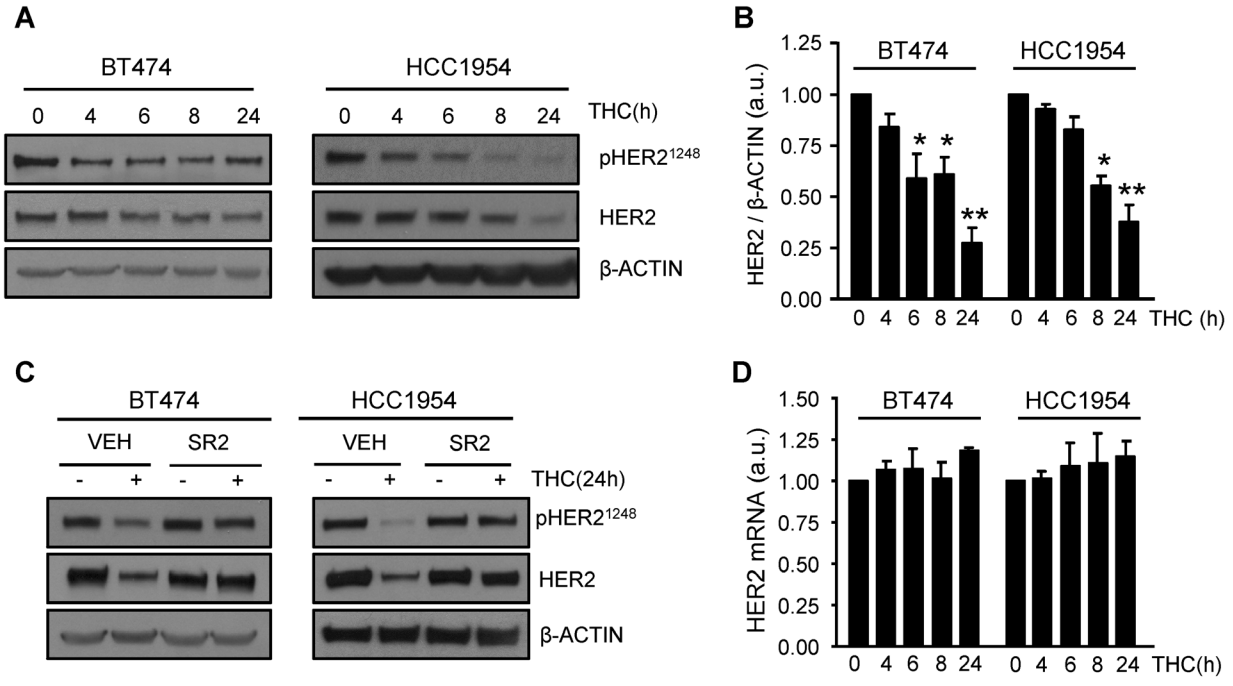


Figure R22. THC induces HER2 degradation *in vitro.*
Effect of THC on HER2 protein **(A, B)** and mRNA levels **(D)** at the indicated times, as determined by Western blot and Q-PCR, respectively, in BT474 and HCC1954 cells. For quantification, HER2 expression was normalized with the loading control [β-Actin in **(B)**, and β-Actin and GUSB in **(D)**], and results (n≥3 independent experiments) expressed as fold increase *vs* time 0, set at 1. (C) Western blot analysis of the effect of the CB2R-selective antagonist SR144528 (SR2, 1μM) on THC-induced HER2 protein decrease. One-way ANOVA with Tukey's post hoc test. *, p<0.05; **, p <0.01, *vs* time 0.

Importantly, THC also produced the disruption of HER2-CB2R heteromers *in vivo*, an effect that was associated to HER2 degradation, and antitumor responses. Thus, THC significantly decreased the growth of orthotopic xenografts generated in immunedeficient mice by injection of HCC1954 cells (Figure R23A), and tumors from the THC-treated group showed significantly reduced HER2 protein levels (Figures R23B-C), as well as significantly reduced HER2-CB2R and HER2-HER2 PLA signal (Figures R23D-E), when compared to vehicle-treated animals.

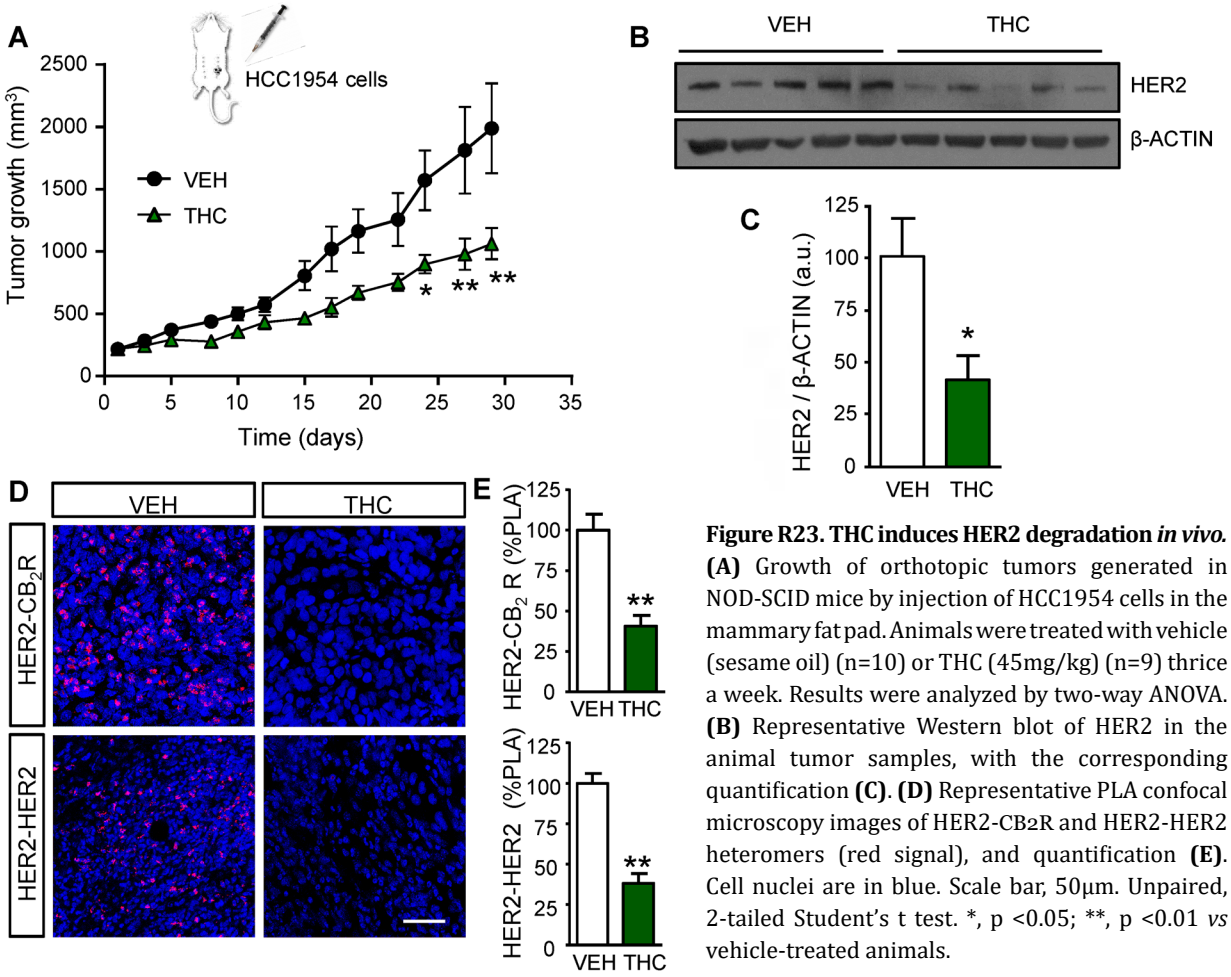


Figure R23. THC induces HER2 degradation *in vivo*. **(A)** Growth of orthotopic tumors generated in NOD-SCID mice by injection of HCC1954 cells in the mammary fat pad. Animals were treated with vehicle (sesame oil) (n=10) or THC (45mg/kg) (n=9) thrice a week. Results were analyzed by two-way ANOVA. **(B)** Representative Western blot of HER2 in the animal tumor samples, with the corresponding quantification **(C)**. **(D)** Representative PLA confocal microscopy images of HER2-CB2R and HER2-HER2 heteromers (red signal), and quantification **(E)**. Cell nuclei are in blue. Scale bar, 50μm. Unpaired, 2-tailed Student's t test. *, p <0.05; **, p <0.01 *vs* vehicle-treated animals.

3.4. THC induces HER2 degradation *via* c-CBL upregulation

As explained in the Introduction, one of the main mechanisms that contributes to HER2 oncogenic signaling is its resistance to internalization and degradation, thus maintaining its pro-tumoral activity at the cell surface for longer periods of time (Bertelsen and Stang 2014). However, it has been described that some anti-cancer drugs such as HSP90 inhibitors (Corina Marx 2010; Wanping Xu *et al.* 2002) or anti-HER2 monoclonal antibodies (Klapper *et al.* 2000) can induce its degradation by the proteasome. Therefore, we next investigated whether the proteasome was involved in THC-induced HER2 degradation. Blockade of the proteasome system with lactacystin prevented the decrease of HER2 levels induced by THC in BT474 breast cancer cells (Figure R24A-B). We performed similar

experiments in HCC1954 cells, but they showed hypersensitive to proteasome inhibition and died in response to minimal concentrations of lactacystin. THC also increased the levels of ubiquitinated HER2 (Figure R24C). The main E3 ligases reported so far to be responsible for HER2 degradation are CHIP and c-CBL (Varshavsky 2017). While cannabinoid treatment did not modify the levels of the

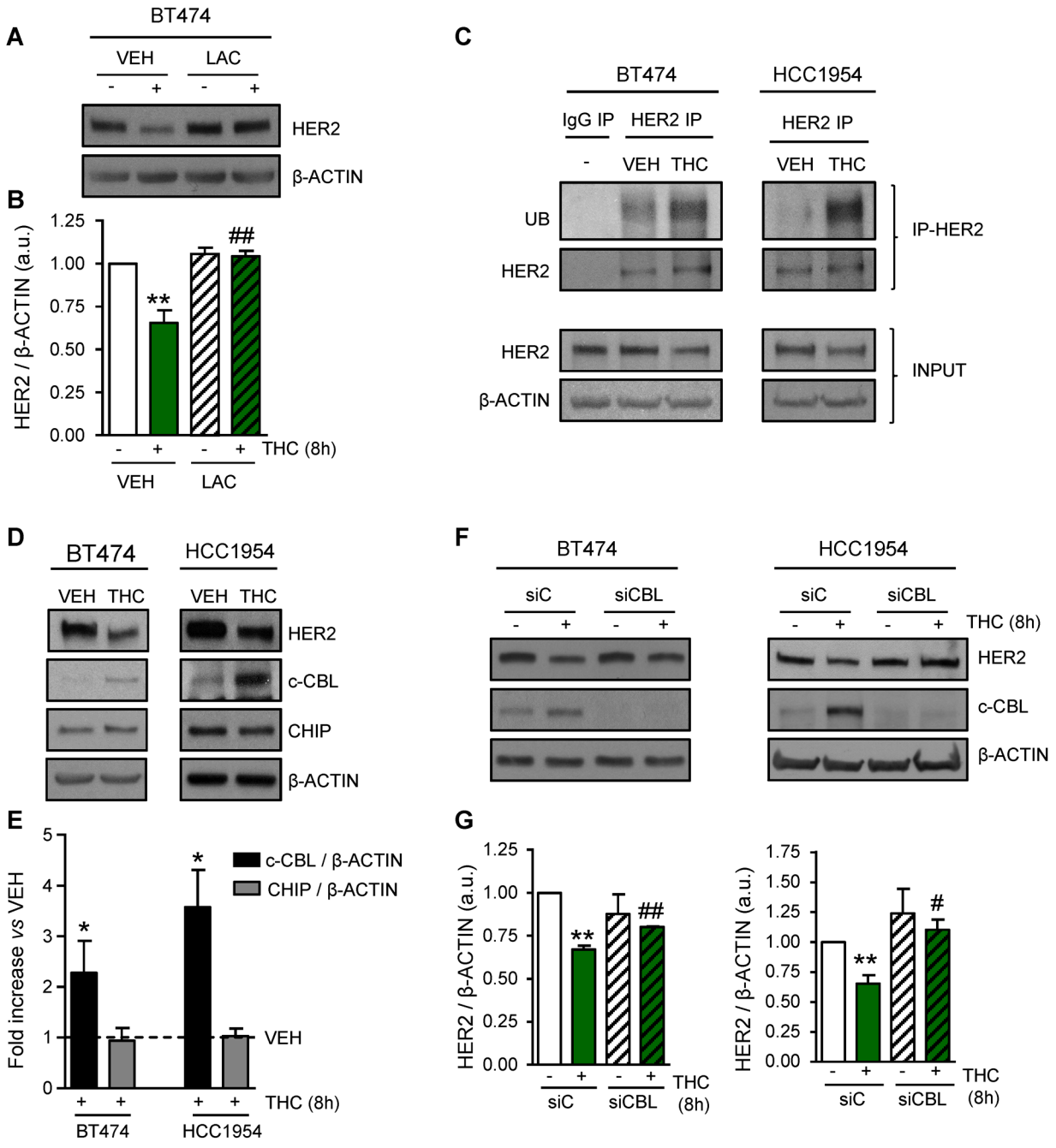


Figure R24. THC induces HER2 degradation *via* c-CBL E3 ligase.
Western blot-based analyses of the effect of different pharmacological and genetic tools on THC-induced HER2 degradation. **(A, B)** Effect of lactacystin (LAC, 1μM) on BT474 cells. Effect of THC (4h) on ubiquitinated HER2 levels **(C)**, or on c-CBL and CHIP levels **(D, E)**, in the indicated breast cancer cell lines. **(F, G)** HER2 protein expression after genetic silencing of c-CBL with a selective siRNA (siCBL). A nontargeted siRNA was used as a control (siC). The densitometric analyses of HER2 immunoblots were normalized to β-Actin **(B, E, G)**. Results from a minimum of 3 experiments are expressed as fold increase *vs* vehicle-treated cells, set at 1. Unpaired, independent groups of 2 were analyzed by 2-tailed Student's t test. When multi-group comparison was required, data were analyzed by One-way ANOVA with Tukey's post hoc test. *, p <0.05; **, p <0.01 *vs* vehicle-treated group; #, p <0.05; ##, p <0.01 *vs* THC-treated group.

former, it significantly increased the amount of c-CBL in BT474 and HCC1954 cells (Figure R24D-E). Involvement of c-CBL in HER2 degradation was further supported by genetic blockade. siRNA-driven targeting of this E3 ligase prevented THC-induced decrease of total HER2 levels in the two breast cancer cell lines tested (Figure R24F-G). Collectively, these findings demonstrate that the disruption of HER2-CB2R heteromers by THC blocks HER2 activation and promotes its degradation through the proteasome system *via* c-CBL activation, which results in antitumor responses.

3.5. HER2-CB2R heteromer disruption by targeting CB2R TM5 mimics THC effects

To confirm that HER2-CB2R heteromer disruption is the responsible for the anti-tumoral effects described above, we used two different experimental approaches aimed at blocking the physical interaction between HER2 and CB2R. First, to determine which part of the cannabinoid receptor is involved in the interaction with HER2, we generated a series of truncated proteins containing the N-terminal domain of CB2R, followed by one of the seven transmembrane (TM) domains of the receptor and its C-terminal domain. All constructs contained an HA tag in the N-terminal domain (Figure R25A). Co-immunoprecipitacion assays showed a potential interaction between HER2 and TMs 1, 3, 4 and 5 of CB2R (Figure R25B). By Bimolecular Fluorescence Complementation (BiFC) assays we found that expression of CB2Rs TM4 and TM5 in HER2nYFP and CB2RcYFP-containing cells prevents the heteromer formation as revealed by loss of fluorescence. Such decrease in complementation signal was not observed when expressing the rest of the CB2R transmembrane mutants, suggesting that TM4 and TM5 are the CB2R regions involved in the interaction with HER2 (Figure R25C-D). Since TM5 has been previously described to be involved in interactions between GPCRs (Borroto-Escuela *et al.* 2010; Franco *et al.* 2016; Gaitonde and González-Maeso 2017), we focused our studies on this specific transmembrane domain. Synthetic peptides with the sequence of TM5 of CB2R and D44R (as negative control), fused to HIV TAT, were then used to prevent the association between CB2R and HER2 and to evaluate the functional consequences. BiFC experiments confirmed that this tool selectively blocks the formation of HER2-CB2R heteromers (Figure R25E). Thus, the fluorescent signal indicative of the presence of HER2-CB2R heteromers disappeared when cells were incubated with the CB2R TAT-TM5 peptide, and not when they were challenged with a D44R TAT-TM5-targeted peptide (used as negative control) (Figure R25E). Similar data were obtained when PLAs were carried out in native untransfected HER2+ breast cancer cells (Figure R26A), *i.e.,* a significant decrease in the dotted fluorescent signal corresponding to the heteromers appeared upon CB2R TAT-TM5 treatment, which was not evident when the D44R TM5 peptide was used (Figure R26A-B). Of interest, and as observed for THC, disruption of HER2-CB2R heteromers by the CB2R TAT-TM5 peptide produced 1) HER2 inactivation, as demonstrated by a dramatic decrease in the formation of HER2-HER2 homodimers (Figure R26C-D), and in the levels of phosphorylated HER2 (Figure R26E); 2) HER2 degradation, evidenced by a marked reduction in total HER2 protein levels (Figure R26E-F); and 3) a concomitant decrease in the viability of HER2+ breast cancer cells (Figure R26G), that was not observed in wild-type HEK293 cells, which do not express either HER2 or CB2R (Figure R6g).

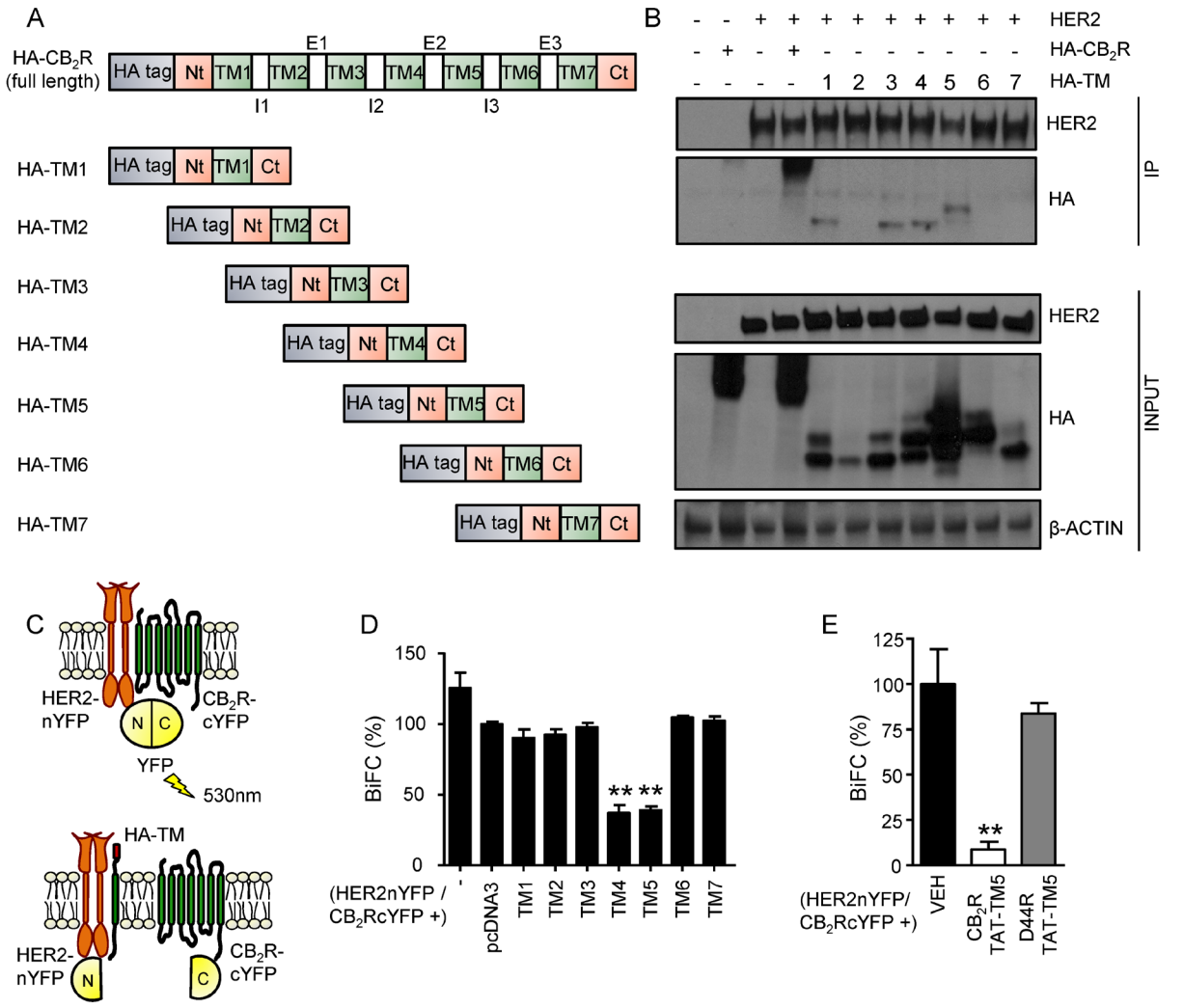


Figure R25. CB2R transmembrane 5 is involved in HER2-CB2R heterodimerization

(A) Schematic representation of the HA-tagged CB2R truncated constructs used in this study. Each construct contains a HA tag, followed by the N-terminal domain of the receptor, one of its 7 transmembrane domains, and the C-terminal end. **(B)** Each of the 7 CB2R constructs (named as HA-TMX, where X is the corresponding transmembrane domain) and a pcDNA3-HER2 plasmid were co-expressed in HEK293 cells. Immunoprecipitation of HER2 with an anti-HER2 antibody was followed by Western blot analysis with an anti-HA antibody. Full length pcDNA3-HA-CB2R was also co-expressed with HER2 as a positive control of interaction. **(C)** Schematic representation of the Bimolecular Fluorescence Complementation (BiFC) experiments between HER2-cYFP and CB2R-nYFP in the absence (upper panel) or in the presence of the CB2R transmembrane constructs (lower panel). **(D, E)** Complementation signal (i.e. fluorescence at 530nm) of HEK293 cells transfected with CB2R-cYFP, HER2-nYFP and the indicatedCB2R TM constructs **(D)**, or after 4h of incubation with the indicated TAT-TM peptides (4μM) **(E)**. Results were analyzed by One-way ANOVA with Tukey's post hoc test. **, p <0.01 *vs* pcDNA3 **(D)** or vehicle-treated group **(E).**

Altogether, these results provide new mechanistic insights into THC antitumoral effects, and more general, introduce HER2-CB2R heteromer disruption as a new strategy for the management of HER2+ breast cancer.

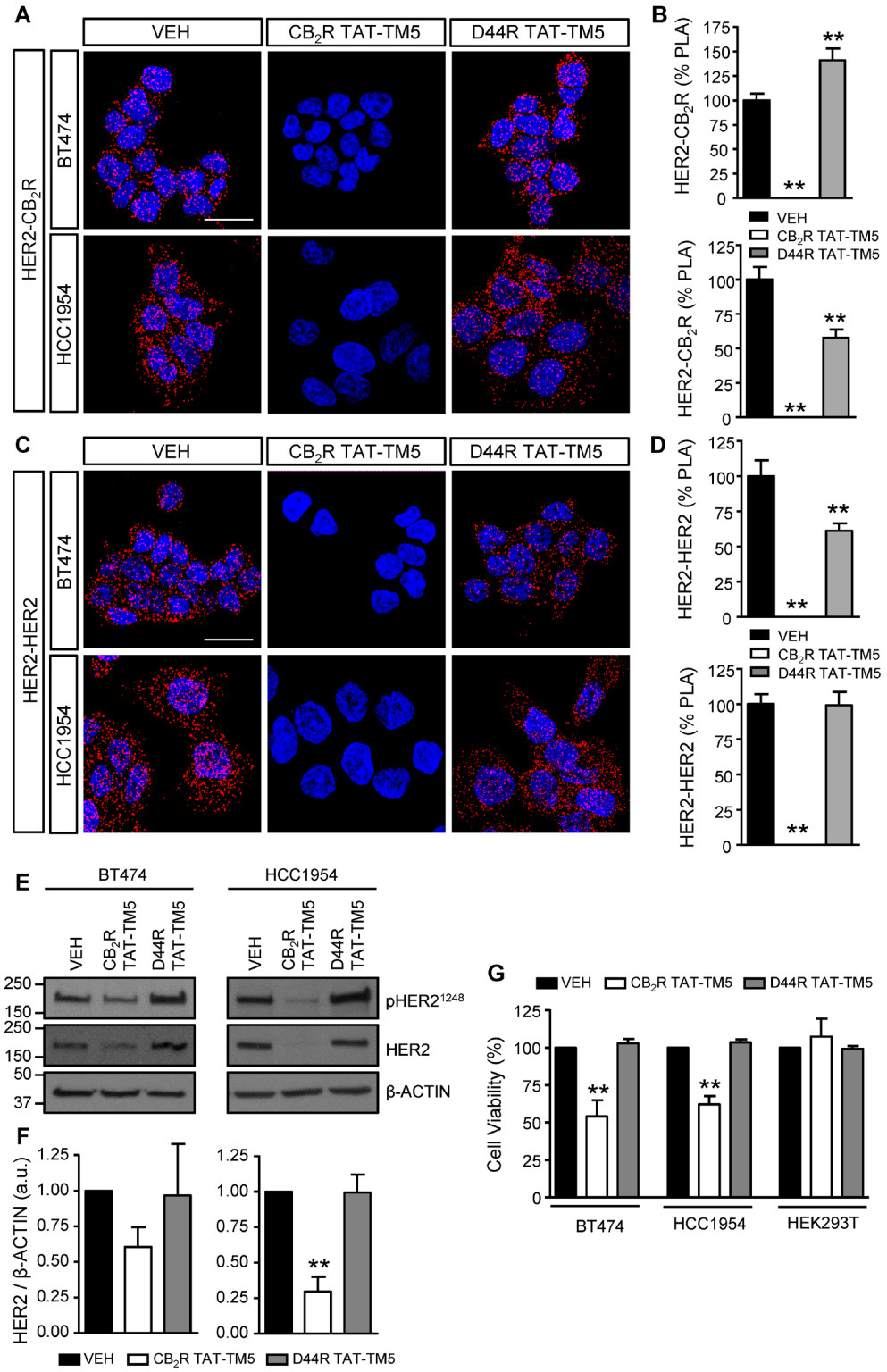
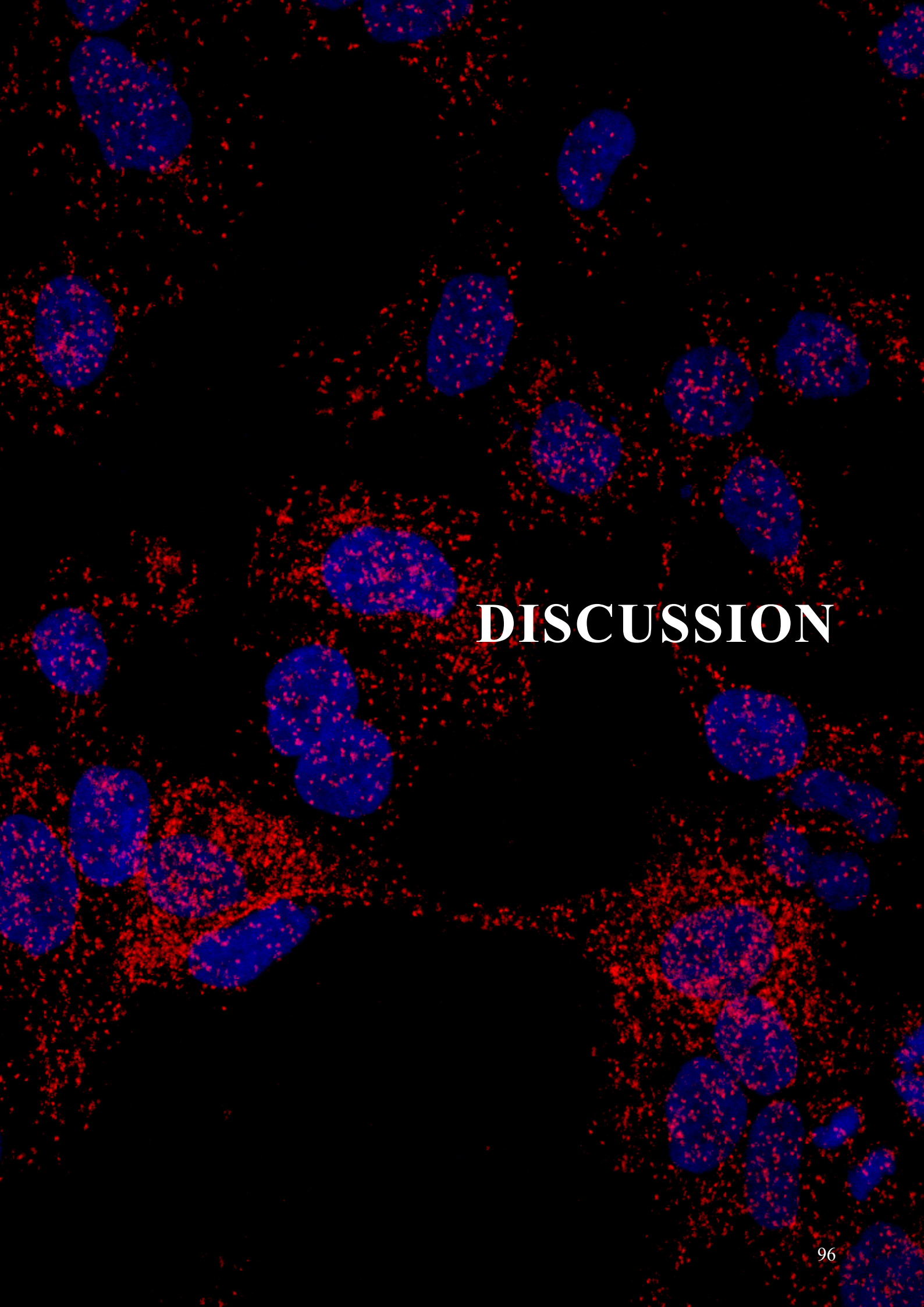


Figure R26. HER2-CB2R heteromer disruption by targeting CB2R TM5 mimics THC effects.
(A-D) Effect of TAT-TM peptides on HER2-CB2R and HER2-HER2 heteromer expression as determined by PLA. **(A, C)** Representative PLA images in the indicated breast cancer cell lines, after treatment for 4h with vehicle (DMSO), a TAT-TM peptide targeting CB2R TM5 (4μM), or a TAT-TM peptide targeting dopamine receptor D44 (4μM), used as a negative control. Dimer signal is in red, and cell nuclei in blue. Scale bars: 25μm. **(B,D)** Quantification of a minimum of 3 experiments. Results are expressed as % of PLA (red dots per cell) *vs* vehicle-treated cells, set as 100%. **(E)** pHER2^{1248} and HER2 protein levels, as determined by Western blot, after treatment with vehicle, CB2R TAT-TM5 or D44R TAT-TM5 peptides for 24h in BT474 and HCC1954 cells. Densitometric analysis of HER2 normalized to β-Actin. Results are represented as fold increase *vs* vehicle-treated cells, set as 1. **(F)** Viability of HCC1954, BT474 and HEK293 cells in response to the indicated treatments for 24h. Data from a minimum of 3 experiments are represented as % *vs* vehicle-treated cells, set as 100%. One-way ANOVA with Tukey's post-hoc test. **, p <0.01 *vs* vehicle-treated cells.



DISCUSSION

Cancer is the second leading cause of death worldwide among women right after cardiovascular diseases. Breast cancer in particular is the most common, accounting for more than 1.5 million cases diagnosed and roughly 500.000 deaths per year (Ferlay *et al.* 2015). The clinical outcome of this patient population as a whole has improved during the last decades mainly due to early diagnosis and better treatments. However, many of these patients present very aggressive tumors that do not respond to current therapies or respond initially but eventually develop resistance. This represents a clinical challenge with two complementary objectives: the design of new antitumor treatments, and the implamentation of new tools to early identify those patients at higher risk of recurrence. To achieve both goals, it is crucial to understand in molecular terms how tumors are produced and how they progress. An enhanced knowledge on this subject will allow the discovery of new therapeutic targets for the treatment of cancer and of new biomarkers with prognostic or predictive value. In this Thesis, we aimed at shedding light on the role of the CB2R cannabinoid receptor in breast cancer generation and progression. Here, describe that CB2R expression is highly associated to HER2+ breast tumors and that CB2R exerts a remarkable pro-oncogenic function in this specific subtype of breast cancer. Moreover, we demonstrate that CB2R and HER2 physically interact, thus forming heteromers, and that their expression correlates with poor patient prognosis. Finally, we show that disruption of these complexes could be therapeutically exploited to promote anti-tumoral responses.

CB2R overexpression has been reported in different types of cancer, but its role in the physiopathology of this disease seems to be very context-dependent. For example, we observe a strong association between higher CB2R protein expression in HER2+ breast tumors and lower relapse-free, metastasis-free and overall patient survival. Interestingly, CB2R upregulation has been correlated with poor patient prognosis in other types of cancer, such as glioma (Wu *et al.* 2012), colorectal cancer (Martinez-Martinez *et al.* 2015), renal cell carcinoma (J. Wang *et al.* 2017) and squamous cell carcinoma of head and neck (Klein Nulent *et al.* 2013). More recently, Elbaz and coworkers reported an upregulation of CB2R in breast cancer compared to healthy tissue, but, in this case, CB2R expression was associated with better patient prognosis (Elbaz *et al.* 2016). Although these results seem to contradict our findings, it is important to highlight that they only analyzed the expression of CB2R in basal and luminal A breast tumors, but not in the HER2+ subtype. Further experiments -using genetic or pharmacological manipulation of the receptor- are needed to unravel whether CB2R upregulation is a cause or a consequence of the disease in each specific tumor type. In our case, we cogently demonstrate that CB2R potentiates HER2 oncogenic activity in breast cancer. Thus, by using a wide variety of cell- and animal-based experimental approaches, we found that CB2R silencing impaired cancer cell proliferation, invasion, tumor growth and metastasis in models of HER2+ breast cancer, whereas it caused no effect in models of HER2-negative breast tumors. These data reveal a pivotal role of CB2R in HER2-mediated pro-oncogenic signaling, and unveil a new mechanism controlling the activity of HER2 that may constitute a new target for antitumor treatments.

We also found a new molecular mechanism that explains the strong association between HER2 and

CB2R expression. Specifically, we observed that HER2 promotes CB2R expression by activating the transcription factor ELK1 *via* MAPK/ERK (Figure D1). This mechanistic model may constitute a novel positive feedback loop to sustain HER2-dependent oncogenic signaling of breast cancer cells. Thus, HER2 promotes CB2R expression, which in turn favors HER2 pro-tumoral signaling. A similar link has also been stablished between other GPCRs and RTKs. For example, HER2 overexpression induces activation of ERK signaling, autocrine release of epinephrine, and, consequently, up-regulation of β2-AR levels (Shi *et al.* 2010).

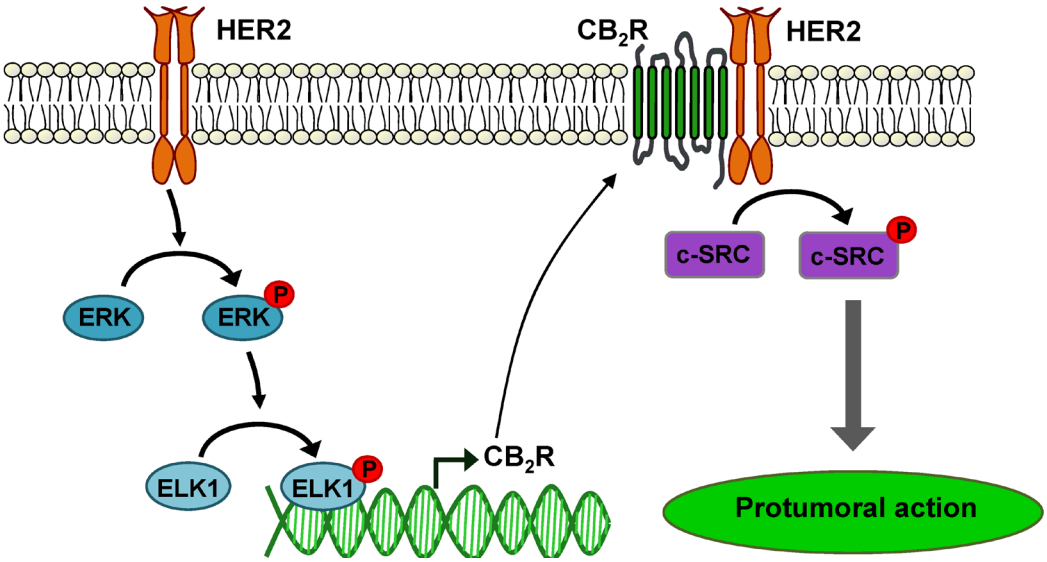


Figure D1. Proposed role of CB2R in HER2-driven pro-oncogenic signaling.
HER2 enhances CB2R expression by activating the transcription factor ELK1 via ERK. Increased CB2R expression promotes HER2 pro-oncogenic signaling by activating the tyrosine kinase c-SRC.

It is widely accepted that GPCRs and RTKs control critical biological processes intimately related to oncogenesis, and that the functional crosstalk between members of these two receptor superfamilies (*e.g.* transactivation of RTKs by GPCR-mediated signaling or *vice versa*) might contribute to the progression and resistance to RTK-targeted therapies of some types of cancer (Pyne and Pyne 2011), including HER2+ breast cancer (Li *et al.* 2004). One of the main mechanisms of GPCR-mediated RTK transactivation involves the activation of non-receptor protein tyrosine kinases. Hence, GPCR-activated members of the SRC family (c-SRC in particular) induce ligand-independent transactivation of RTKs *via* trans-phosphorylation of cytosolic tyrosines, which provide docking sites for adaptor proteins that trigger different intracellular signaling cascades (Cattaneo *et al.* 2014). Our findings show that c-SRC is involved in CB2R-induced HER2 pro-oncogenic signaling. c-SRC promotes cell proliferation, survival, migration and angiogenesis (Yeatman 2004), and its deregulation is associated with oncogenesis (Kim *et al.* 2009; Zhang and Yu 2012) and poor patient prognosis (Wheeler *et al.* 2009). Together, these features make c-SRC a potential target for the clinical development of specific

inhibitors (Aleshin and Finn 2010). In HER2+ breast cancer in particular, c-SRC activation has been implicated in the generation of brain metastases (Zhang *et al.* 2013), a condition that has no curative treatment, and in the development of trastuzumab resistance (Zhang *et al.* 2011). Interestingly, treatment of brain metastasis-bearing mice with a combination of lapatinib (which targets HER1 and HER2) and a c-SRC inhibitor slowed-down the growth of the metastases (Zhang *et al.* 2013), and treatment of trastuzumab-resistant cells/tumors with a c-SRC inhibitor restored trastuzumab resistance (Zhang *et al.* 2011). These data suggest that blocking c-SRC may be an effective approach to treat two important remaining clinical challenges in HER2+ breast cancer: the management of highly metastatic tumors (especially those colonizing the central nervous system) and trastuzumab resistance (both innate and acquired).

The functional crosstalk between GPCRs and RTKs might rely in some cases on a physical interaction between receptors. Here we show for the first time that HER2 forms heteromers with a GPCR (CB2R) in cancer cells. In particular, the HER2-CB2R heteromers described in this study fulfill the three criteria required for demonstrating receptor heteromerization (Gomes *et al.* 2016): first, the heteromer components (HER2 and CB2R) interact physically in native cells, as demonstrated by colocalization and proximity-based experiments performed in human HER2+ breast tumors; second, HER2-CB2R heteromers exhibit properties distinct from those of the protomers, as demonstrated by the coupling of CB2R to different heterotrimeric G proteins depending on whether it is part of the heteromer or not; and third, heteromer disruption leads to a loss of heteromer-specific properties, as demonstrated by the fact that while HER2-CB2R complexes are linked to pro-oncogenic events, the disruption of the heteromers leads to antitumor responses.

Interaction of HER2 with other RTKs is a common and well described process. Dimerization with other members of the HER family, for example, is a necessary step for HER activation, and in fact some drugs have been already designed to interfere with this process and block the subsequent pro-oncogenic signaling (Figure 5 of Introduction). An increasing number of studies demonstrate that GPCRs also interact physically between them, generating unique signaling platforms (GPCR heteromers). Although several RTK-RTK heteromers and GPCR-GPCR heteromers have been previously described, there are very few examples of physical interactions between RTKs and GPCRs yet. Transactivation of RTKs by GPCRs and *vice versa* has been reported, and in some cases physical interactions suggested, but no solid proof of the existence of such heteromers has been provided in most cases (Pyne and Pyne 2011). To the best of our knowledge, the most solidly reported RTK-GPCR heteromer is that formed by HER2 and the β2-adrenergic receptors in the heart (Negro *et al.* 2006); by fibroblast growth factor and adenosine A_{2A} receptors (Flajolet *et al.* 2008) or serotonin 5-HT_{1A} receptors (Borroto-Escuela *et al.* 2012), and by EGFR and GPR54 (Zajac *et al.* 2011). Here, we comprehensively describe the existence of a new heteromer between HER2 and a GPCR (CB2R), and provide compelling evidence showing that these novel complexes are associated to poor patient prognosis. All these observations may serve to exploit the HER2-CB2R heteromer as a new prognostic

marker in oncology (at least in certain types of tumors). In fact, it would be interesting to design new methods to detect and quantify these heteromers in human samples. For example, anti-HER2-CB2R-selective antibodies would allow not only corroboration of the prognostic value described herein but also easy transfer of this knowledge to the clinical practice (Gomes *et al.* 2014). Moreover, in cases in which the heteromer expression is upregulated, such as HER2+ breast cancer, these selective antibodies or similar tools might be useful for blocking the heteromer-induced biological responses, and thus could be used as targeted antitumoral therapeutic agents.

Here, we also provide compelling evidence that disruption of HER2-CB2R heteromers produces antitumor responses. First, we found that activation of CB2R by THC induces the disruption of HER-CB2R heteromers, which triggers the inactivation (by breaking HER2-HER2 homodimers) and degradation of HER2, thereby promoting antitumor responses. Based on these observations, we proposed that disrupting HER2-CB2R complexes may constitute a new general strategy to treat HER2+ breast tumors. To test this hypothesis, we first aimed at elucidating the protein domains involved in HER2-CB2R heterodimerization. Although previous studies have shed some light on the receptor regions involved in the interaction between cannabinoid receptors and other GPCRs (Viñals *et al.* 2015; Moreno *et al.* 2017), very little is known about the putative domains of CBRs involved in the interaction with RTKs (Borroto-Escuela *et al.* 2016). Our findings show that CB2R TM5 is required for HER2-CB2R heteromerization. Thus, by using a membrane-permeable peptide with an identical sequence to CB2R TM5, we were able to break HER2-CB2R heteromers and mimic THC-evoked effects (*i.e.* HER2 inactivation, HER2 degradation, and cancer cell death). These findings not only support that HER2-CB2R heteromers might be potential targets for new HER2+ breast cancer therapies, but also highlight the importance of the HER2-CB2R interaction in the maintenance of HER2 activation and stabilization. Thus, by breaking this interaction with different approaches, we found that HER2 homodimerization is impaired, resulting in HER2 inactivation and degradation by the proteasome. It is therefore tempting to speculate that CB2R could indirectly act as a chaperone, protecting HER2 from degradation. Another plausible explanation is the presence of other proteins in the HER2-CB2R complex that participate in HER2 stabilization, such as HSP90 (Calderwood *et al.* 2006). To test these hypotheses, further experiments aimed at evaluating the effect of CB2R silencing on HER2 activation and degradation, and identifying additional proteins in the complex, should be performed.

Although we have focused our research in breast cancer, it is conceivable that other HER2-overexpressing tumors such as gastric or gastroesophageal adenocarcinomas (Mishra *et al.* 2017) may express similar HER2-CB2R heteromers, and therefore respond in a similar way to treatments aimed at breaking up these complexes. Following the same rationale, CB2R could interact with other RTKs different than HER2 in other tumor types. Of note, antitumor responses upon CB2R activation have also been described in non-HER2+ contexts. It would be interesting to analyze whether in those situations CB2R acts as a monomer in the plasma membrane or it forms heteromers with other RTKs

like HER1 (EGFR), which is overexpressed in many types of tumors (Mishra *et al.* 2017). In favor of the latter, it has been reported that CB2R impairs oncogenic EGF/EGFR signaling in ER+ breast cancer cells (Elbaz *et al.* 2016). Although not proved, the authors suggested that EGFR and CB2R might form complexes, and that CB2R activation might disrupt them. In addition, and in line with our data, pharmacological activation of CB1R induced the death of prostate cancer cells in culture, an effect that was accompanied by a significant downregulation of EGFR (Mimeault *et al.* 2003), and co-expression of EGFR with CB1R was associated to poor patient prognosis in this type of cancer (Fowler, Hammarsten, and Bergh 2010). These observations demonstrate a functional interaction between another cannabinoid receptor (CB1R) and another member of the HER family (HER1), that could be due to a mere transactivation processes or to a physical interaction similar to that described here for CB2R and HER2.

One of the main conclusions of this work is that targeting HER2-CB2R heteromers, either with THC or with other tools aimed at disrupting the interaction between the two receptors, might be used as a new antitumoral strategy in HER2+ breast cancer management. The therapeutic potential and safety of cannabinoids-based medicines have been proved in clinical trials for various applications in cancer patients [*e.g.* inhibition of nausea, vomiting and pain in cancer patients (Abrams & Guzman, 2015)]. In fact, capsules of THC, named dronabinol (Marinol®), and its synthetic analogue nabilone (Cesamet®), have been approved by the FDA to alleviate nausea and vomiting induced by chemoterapy in cancer patients. Additionally, a standardized cannabis extract, nabiximols (Sativex®), has been approved by Health Canada for the treatment of cancer-associated pain. Moreover, there is indirect evidence that cannabinoid-based medicine can be safely combined with standar anticancer treaments. Thus, tens of clinical trials have been performed in cancer patients that were undergoing their classical treatments and concomitantly received cannabinoid drugs to test their efficacy as antiemetic or analgesic tools. No negative interactions between treatments have been reported so far. In addition, and at least at the preclinical level, the combination of cannabinoids with other anticancer therapies increases the antitumor efficacy of the corresponding individual treatments (Miyato *et al.* 2009; Torres *et al.* 2011; Velasco *et al.* 2012). In the specific context of HER2+ breast cancer, results from our laboratory show that combination of THC with HER2-targeted therapies produces additive anti-proliferative response in cell cultures (Blasco-Benito *et al.* 2018). Similar results were previously reported by the combination of cannabinoids and temozolamide (an alkylating agent that is the standard treatment of glioblastoma multiforme) (Torres *et al.* 2011), which set the basis for the first controlled clinical study aimed at evaluating the safety and efficacy of Sativex (a cannabis extract containing equal amounts of THC and CBD) combined with temozolamide in patients with recurrent glioblastoma multiforme (ClinicalTrials.gov identifiers NCT01812603 and NCT01812616). Although the results have not been published yet, a press release from the sponsoring company has unveiled positive results (https://www.gwpharm.com).

The use of membrane-permeable peptides to hamper heteromer formation, represents a significant advance in our ability to access, interrogate and manipulate complexes such as HER2-CB2R heteromers, and it may also represent a new which might be a new antitumoral therapeutic strategy. In line with this idea, peptides mimicking the transmembrane domain of HER2 have been shown to specifically antagonize this receptor, thus inhibiting its phosphorylation and downstream signaling, and thereby evoking cancer cell death and reduction of tumor growth and metastasis (Arpel *et al.* 2014; Bublil *et al.* 2016). It would be interesting to study whether those peptides also disrupt HER2-CB2R heteromers, which might thus contribute to their antitumor activity. In further support of this potential therapeutic approach strategy, t is conceivable that specific targeting of HER2-CB2R overexpressing cancer cells might reduce the adverse effects reported for standard anti-HER2 therapies. Moreover, it is also tempting to speculate that conjugation of HER2-CB2R-disrupting peptides with cytotoxic drugs may produce enhanced therapeutic responses in HER2-CB2R overexpressing tumors.

Considering all the results presented in this Thesis, we propose the following model for CB2R function in HER2+ breast cancer (Figure D2): HER2 upregulates the expression of CB2R *via* the mechanisms described in Aim 1 (Figure D1). CB2R then form heteromers with HER2 at the plasma membrane, thereby protecting HER2 from degradation and favoring its canonical oncogenic signaling, which evokes protumoral responses (Figure D2A). When cells are exposed to THC (or to other tools that prevent HER2-CB2R interaction), the two receptors phisically separate. In addition, HER2-CB2R disruption triggers inactivation of HER2 (by breaking HER2-HER2 homodimers) and increases its susceptibility to degradation. As a final consequence of HER2 degradation and CB2R activation, an antitumor response is produced (Figure D2B).

In summary, these findings unveil an unprecedented mechanism of control of HER2 activity, demonstrate that CB2R is an indispensable component of the HER2 pro-oncogenic machinery, and support HER2-CB2R heteromers as new prognostic markers and therapeutic targets in HER2+ breast cancer. Although THC efficiently achieves heteromer disruption, our data set the bases for the design of more selective antitumor drugs or biologicals aimed at breaking this interaction.

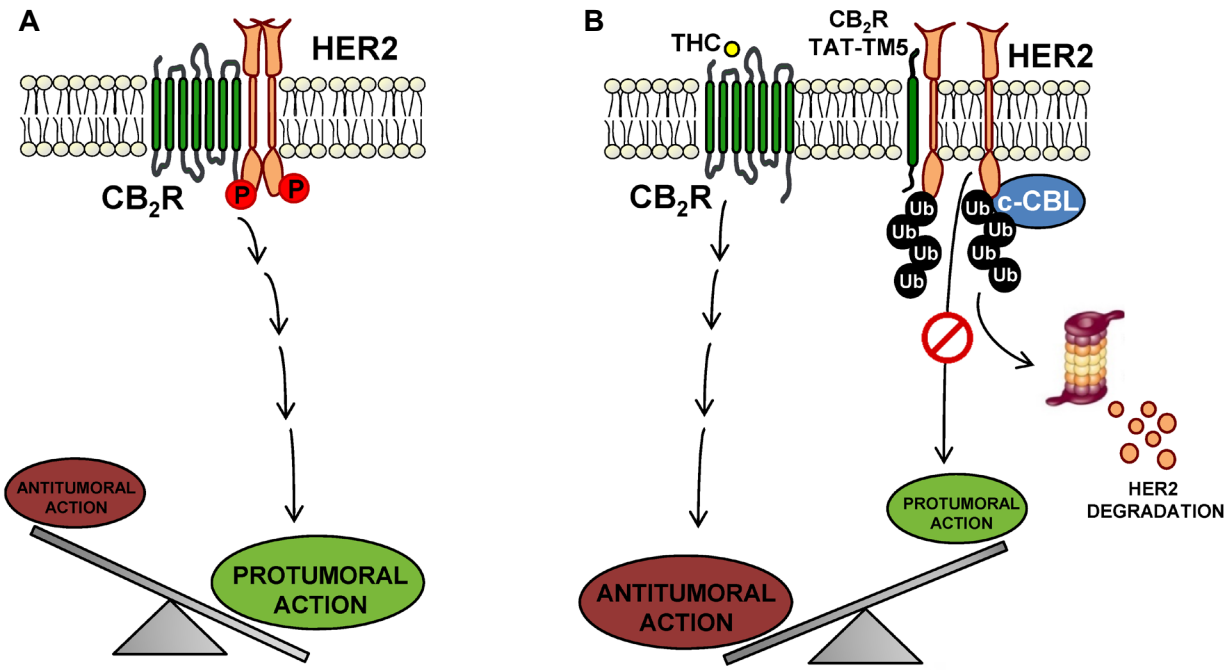


Figure D2. Schematic drawing of the proposed mechanism of control of HER2 function by CB2R.
(A) HER2 and CB2R form heteromers at the plasma membrane, protecting HER2 from degradation and favoring its pro-oncogenic signaling. **(B)** Disruption of HER2-CB2R heteromers, either by THC or by specific tools targeting CB2R transmembrane domain 5, triggers inactivation of HER2 by inducing the separation of HER2-HER2 homodimers, increasing HER2 susceptibility to degradation. HER2 degradation and CB2R activation result in antitumor responses.

CONCLUSIONS

The data obtained in this Doctoral Thesis allow delineating the following conclusions:

1. CB2R is overexpressed in HER2+ breast cancer, and higher levels of the receptor correlate with poor patient prognosis, specifically local relapse free, metastasis-free and overall survival.

2. CB2R overexpression is driven by HER2 by activation of the transcription factor ELK1 *via* ERK, and confers pro-oncogenic properties on breast cancer cells by activating HER2 signaling *via* the non-receptor tyrosine kinase c-SRC.

3. HER2 and CB2R form heteromers with unique signaling properties in HER2+ tumors, and CB2R transmembrane domain 5 is essential for HER2-CB2R interaction. Higher HER2-CB2R heteromer expression is associated to poor patient prognosis in HER2+ breast cancer, specifically lower disease-free and overall survival.

4. Disruption of HER2-CB2R heteromers (either with THC or with tools aimed at preventing HER2-CB2R interaction) produces antitumor responses *in vitro* and *in vivo* by inactivating HER2, and stimulating its proteasomal degradation *via* the E3-ligase c-CBL.

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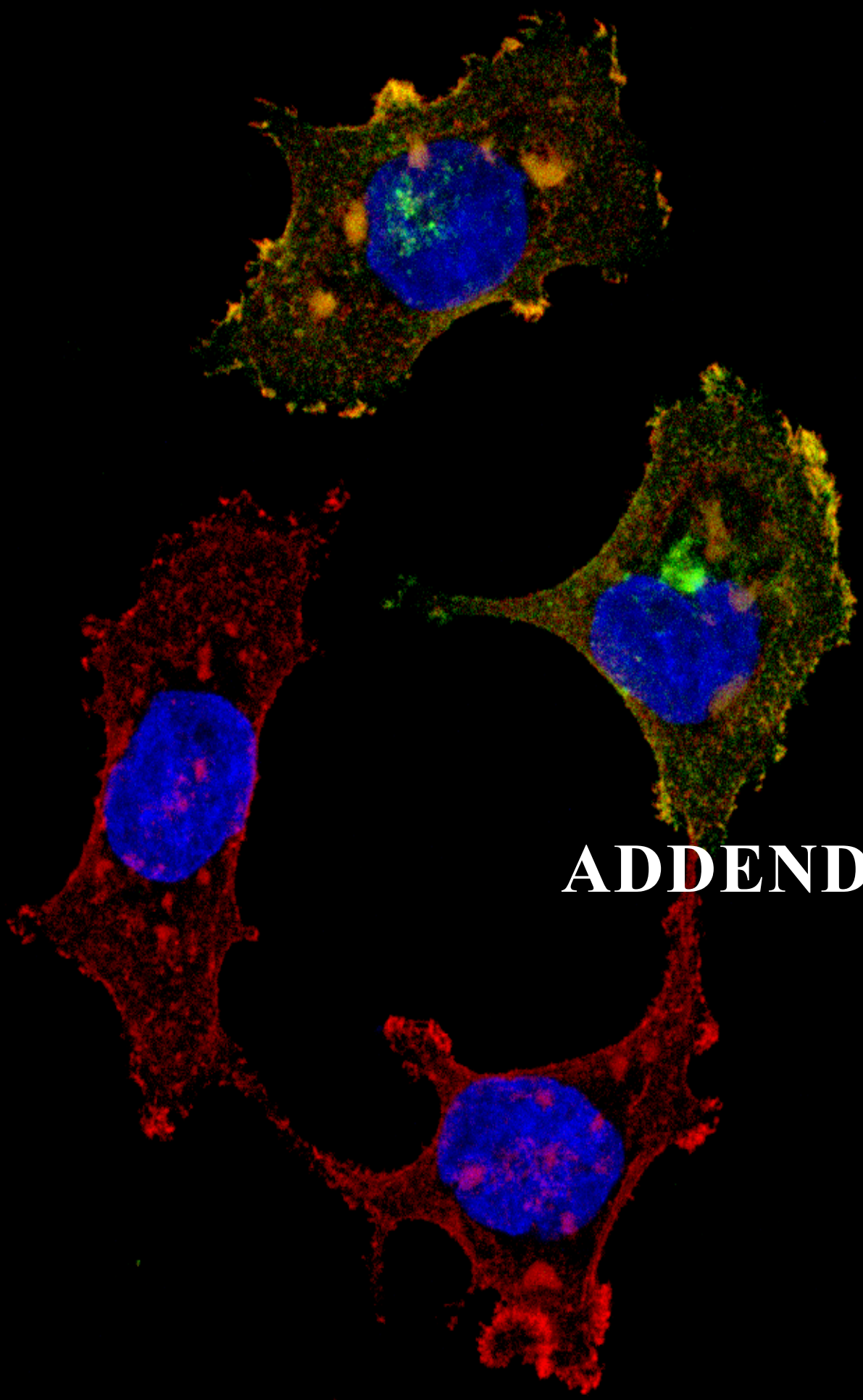
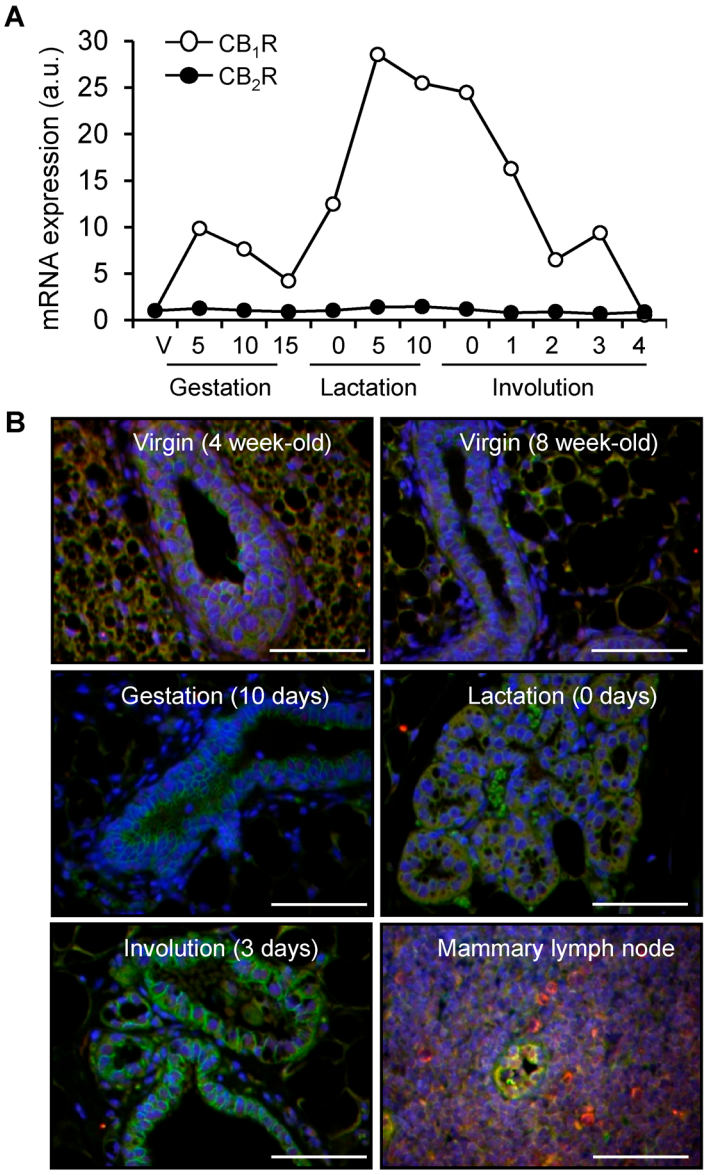
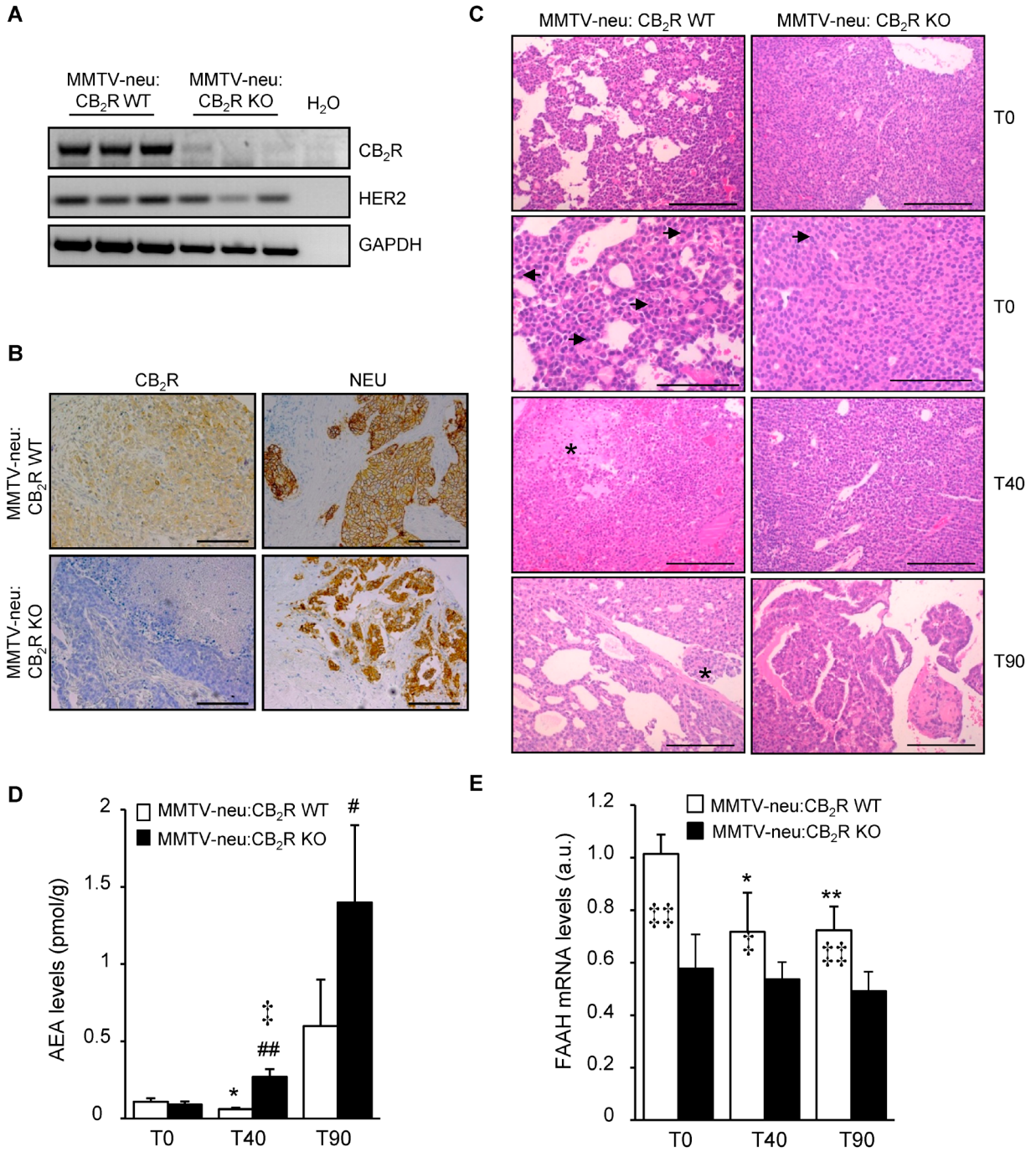


ADDENDA

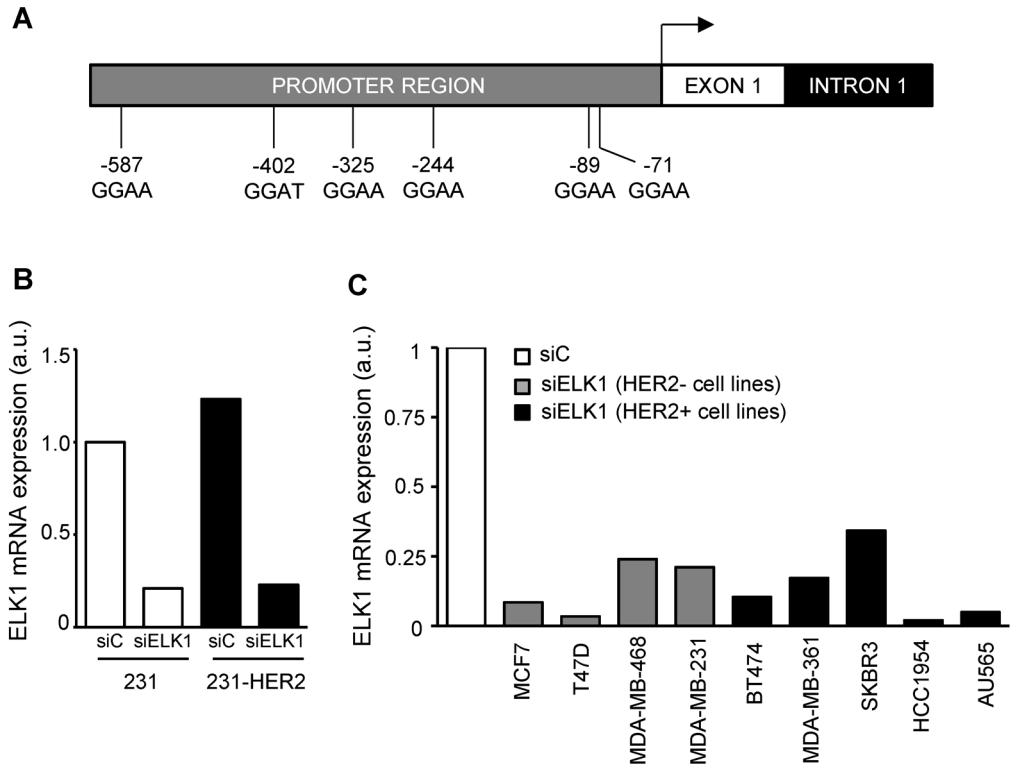
SUPPLEMENTARY FIGURES



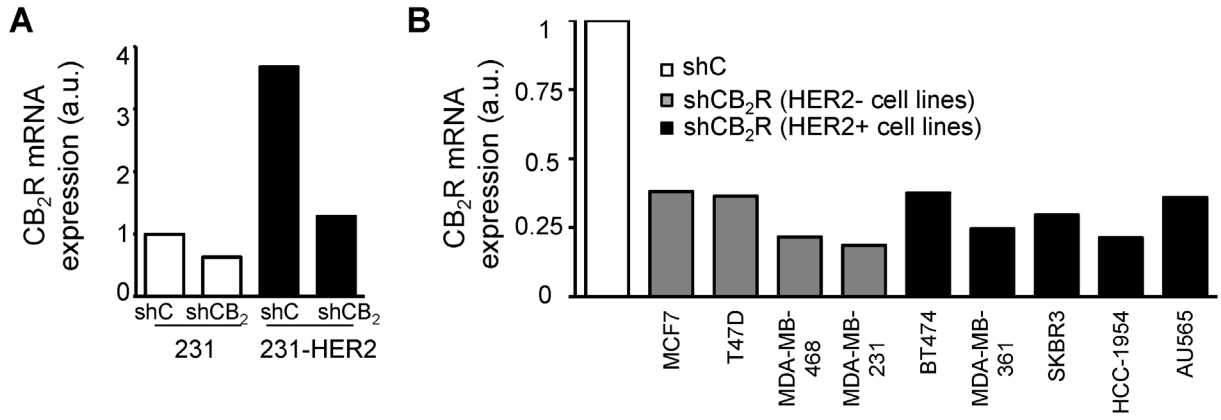
Supplementary Figure 1. Analysis of non-transformed mouse mammary glands. (A) Analysis of CB1R and CB2R mRNA expression in non-cancerous mammary glands of adult wild-type C57Bl/6 mice at the indicated time points of the pregnancy cycle. Results are expressed in arbitrary units. V, 8 week-old virgin mice. Time is expressed in days. **(B)** Immunofluorescence staining of CB2R (red) in representative tissue sections of the different stages of the adult mammary gland development. E-Cadherin (green) staining was performed for epithelial cell visualization and cell nuclei were stained in blue. A mammary lymph node is shown as a positive control of CB2R expression. Scale bar, 100μm.



Supplementary Figure 2. Mice tumor characterization. (A-B) CB2R and HER2 mRNA [as determined by RT-PCR **(A)**] and protein expression [as determined by immunohistochemical analysis **(B)**] in representative tumor samples obtained from MMTV-neu: CB2R WT and MMTV-neu: CB2R KO mice. Scale bars, 200 μm. **(C)** H&E staining of tumour sections obtained at tumour arousal (T0) or forty (T40) or ninety (T90) days after tumor appearance. Arrows point to cells undergoing mitosis. * in WT T40 shows a necrotic area, and in WT T90 a lymphatic invasion. Scale bars, 200 μm except in T0 lower panels (100 μM). mitosis. * in WT T40 shows a necrotic area, and in WT T90 a lymphatic invasion. **(D-E)** Anandamide (AEA) levels [as determined by LC-MS **(D)**] and fatty acid amidohydrolase (FAAH) mRNA levels [as determined by real-time quantitative PCR **(E)**] in tumor samples from the indicated animals at the indicated time points (n≥6 per experimental group and time point). Results are expressed in arbitrary units. Data were analyzed by ANOVA with a post-hoc analysis by the Student–Newman–Keuls' test. All statistical tests were two-sided. *, p< 0.05 and **, p< 0.01 vs MMTV-neu:CB2R WT T0; #, p< 0.05 and ##, p< 0.01 vs MMTV-neu:CB2R KO T0; ‡, p< 0.05 and ‡‡, p< 0.01 vs the corresponding WT.



Supplementary Figure 3. (A) Schematic representation of the CB2R gene promoter sequence. The positions and sequences of the putative ELK1-binding sites are indicated. **(B-C)** ELK1 mRNA expression (as determined by Q-PCR) in MDA-MB-231 (231) (white bars) and MDA-MB-231-HER2 cells (231-HER2) (black bars) **(B)** and HER2- (grey bars) and HER2+ (black bars) breast cancer cell lines **(C)** upon transient transfection with a siRNA selectively targeting ELK1 (siELK1). Results are expressed in arbitrary units *vs* the corresponding siC transfected cells, set at 1.



Supplementary Figure 4. CB2R mRNA expression (as determined by Q-PCR) in MDA-MB-231 (231) (white bars) and MDA-MB-231-HER2 cells (231-HER2) (black bars) **(A)** and HER2- (grey bars) and HER2+ (black bars) breast cancer cell lines **(B)** after stable transfection with a shRNA selectively targeting CB2R (shCB2R). Results are expressed in arbitrary units *vs* the corresponding shC transfected cells, set at 1.

ARTICLES PUBLISHED THROUGHOUT THIS THESIS

Moreno, E., Andradas, C., Medrano, M., Caffarel, M. M., Pérez-Gómez, E., **Blasco-Benito, S**., … Sánchez, C. (2014). Targeting CB2-GPR55 receptor heteromers modulates cancer cell signaling. *Journal of Biological Chemistry, 289*(32), 21960–21972.

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ARTICLE

Role of Cannabinoid Receptor CB_2 in HER2 Pro-oncogenic Signaling in Breast Cancer

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Abstract

Background: Pharmacological activation of cannabinoid receptors elicits antitumoral responses in different cancer models. However, the biological role of these receptors in tumor physio-pathology is still unknown.

Methods: We analyzed CB_2 cannabinoid receptor protein expression in two series of 166 and 483 breast tumor samples operated in the University Hospitals of Kiel, Tübingen, and Freiburg between 1997 and 2010 and CB_2 mRNA expression in previously published DNA microarray datasets. The role of CB_2 in oncogenesis was studied by generating a mouse line that expresses the human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2) rat ortholog (neu) and lacks CB_2 and by a variety of biochemical and cell biology approaches in human breast cancer cells in culture and in vivo, upon modulation of CB_2 expression by si/shRNAs and overexpression plasmids. CB_2-HER2 molecular interaction was studied by colocalization, coimmunoprecipitation, and proximity ligation assays. Statistical tests were two-sided.

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Results: We show an association between elevated CB_2 expression in HER2+ breast tumors and poor patient prognosis (decreased overall survival, hazard ratio [HR] = 0.29, 95% confidence interval [CI] = 0.09 to 0.71, P = .009) and higher probability to suffer local recurrence (HR = 0.09, 95% CI = 0.049 to 0.54, P = .003) and to develop distant metastases (HR = 0.33, 95% CI = 0.13 to 0.75, P = .009). We also demonstrate that genetic inactivation of CB_2 impairs tumor generation and progression in MMTV-neu mice. Moreover, we show that HER2 upregulates CB_2 expression by activating the transcription factor ELK1 via the ERK cascade and that an increased CB_2 expression activates the HER2 pro-oncogenic signaling at the level of the tyrosine kinase c-SRC. Finally, we show HER2 and CB_2 form heteromers in cancer cells.

Conclusions: Our findings reveal an unprecedented role of CB_2 as a pivotal regulator of HER2 pro-oncogenic signaling in breast cancer, and they suggest that CB_2 may be a biomarker with prognostic value in these tumors.

The classical and the most recent molecular classification of breast cancer recognizes a specific entity characterized by the overexpression of the tyrosine kinase receptor (TKR) human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2) (1–4). Activation of TKRs turns on key signaling pathways involved in cell proliferation, development, differentiation, and angiogenesis, among other processes (5). HER2 gene amplification/protein overexpression is detected in 20% to 30% of primary breast cancers and is a predictor of poor prognosis and deficient response to chemotherapy (6).

The endocannabinoid system (ECS) is a cell communication system that participates in the control of different physiological functions such as pain perception, motor behavior, and food intake, just to mention a few (7,8). It consists of two cannabinoid-specific G protein–coupled receptors (GPCRs), CB_1 and CB_2, their endogenous ligands, and the enzymes that produce and metabolize these ligands (7,8). A large number of studies demonstrate that the pharmacological activation of the ECS by different strategies (eg, activation of cannabinoid receptors, inhibition of endocannabinoid degradation) leads to antitumoral responses (7–11). Additionally, it has been shown that the ECS is deregulated in a variety of cancers (7,12–14). Although strong evidence points to the cannabinoid receptor CB_2 as a drug target for antitumoral therapy in several types of cancer (11,14,15), there is no information on its role in tumor generation and progression. Here we show an unprecedented pro-oncogenic role of the cannabinoid receptor CB_2 in HER2+ breast cancer and unveil that this GPCR is a pivotal regulator of HER2 signaling.

Methods

Tissue Microarrays

PFA-fixed and paraffin-embedded blocks of tumor tissue from cases operated in the University Hospitals of Kiel, Tübingen, or Freiburg between 1997 and 2010 were used for tissue microarray (TMA) construction. All patients gave informed consent, and the study was authorized by the respective Hospital Ethics Committees. TMAs were generated by punching two 1 mm spots of each patient's sample. This resulted in two series of 166 and 483 tumor samples. Complete histopathological information was available for all the patients. Additionally, for the 483-sample series (TMA #2), date and cause of death as well as date of local and/or distant relapse were also available.

Immunohistochemical Analysis

Tissue sections were subjected to a heat-induced antigen retrieval step prior to exposure to an anti-CB_2 receptor or an anti-ERBB2 primary antibody (Supplementary Table 1, available online). Immunodetection was performed using the Envision method with DAB as the chromogen. For CB_2 expression, cases were scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (high staining). ERBB2 staining was scored by one independent pathologist in each institution (University Hospitals of Kiel, Tübingen, or Freiburg) in accordance with HercepTest manufacturer's guidelines.

Generation of MMTV-neu:$CB_2^{-/-}$ Mice and Sample Collection

All procedures involving animals were performed with the approval of the Complutense University Animal Experimentation Committee according to the EU official regulations. Generation of the congenic strain MMTV-neu:$CB_2^{-/-}$ was accomplished by mating MMTV-neu mice with $CB_2^{-/-}$ mice (see the Supplementary Methods, available online). Females were palpated twice weekly for mammary gland nodules. As soon as tumors appeared, they were routinely measured with external caliper, and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$. Animals were sacrificed and mammary glands, breast tumors, and lungs were collected (see the Supplementary Methods, available online) at the following time points: 1) when the first tumor in each animal appeared, 2) 40 days after the appearance of the first tumor, and 3) 90 days after the appearance of the first tumor.

Statistical Analysis

The Pearson's chi-squared test was used for statistical analysis of the human samples included in the TMAs. Kaplan-Meier survival curves were statistically compared by the log-rank test. Analysis of variance (ANOVA) with a post hoc analysis by the Student-Newman-Keuls' test was routinely used for the rest of the analyses. Unless otherwise stated, data are expressed as mean ± SD. All statistical tests were two-sided, unless otherwise specified. A P value of less than .05 was considered statistically significant.

Additional methods are available in the Supplementary Methods (available online).

Results

Prognostic Relevance of Tumor CB_2 Expression

In two small cohorts of human samples, we previously reported that CB_2 mRNA expression is associated with higher histological grades and increased HER2 expression (16) and that the CB_2 protein was present in the vast majority of HER2+ tumors (17). Here, we analyzed CB_2 expression in a much larger series of tissue sections (649 breast human samples included in different tissue microarrays [TMAs]). CB_2 expression was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (high staining) (Figure 1A). We observed that nontumor

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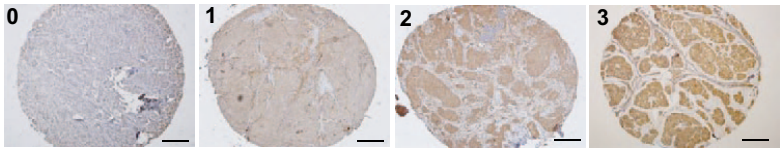

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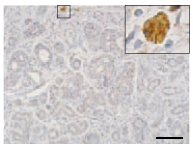

C

		n	CB_2 staining 0	1	2	3	P
Hormone receptor status	Positive	504	117 (23.2%)	191 (37.9%)	135 (26.8%)	61 (12.1%)	.66
	Negative	117	24 (20.5%)	41 (35.0%)	38 (32.5%)	14 (12.0%)	
HER2 status	Positive	92	3 (3.3%)	29 (31.5%)	39 (42.4%)	21 (22.8%)	**<.001**
	Negative	360	86 (23.9%)	141 (39.2%)	94 (26.1%)	39 (10.8%)	
Triple negative status	Positive	47	13 (27.6%)	20 (42.6%)	12 (25.5%)	2 (4.3%)	.14
	Negative	405	76 (18.8%)	150 (37.0%)	121 (29.9%)	58 (14.3%)	

Figure 1. Analysis of CB_2 protein expression in human breast cancer samples. **A)** Representative images showing CB_2 expression scoring according to intensity staining in tissue microarray (TMA) samples: scores 0, 1, 2, and 3 correspond to no, low, moderate, and high staining, respectively. **Scale bar** = 500 μm. **B)** Representative CB_2 immunohistochemical staining in a human nontumor breast tissue sample included in the analyzed TMAs. Inset, CB_2 staining (**brown**) in a macrophage is shown as a CB_2 staining-positive control. **Scale bar** = 200 μm. **C)** Association between CB_2 expression (as determined by staining scoring) and the molecular features of breast tumor samples included in the TMAs. The Pearson's chi-squared test was used for statistical analysis. All statistical tests were two-sided.

breast tissue expressed undetectable levels of CB_2 (Figure 1B). Conversely, CB_2 was expressed by a very large fraction of human breast adenocarcinomas (476 out of 629, ie, 75.6%). CB_2 expression was highly associated to HER2+ tumors ($P < .001$) (Figure 1C), while no association between CB_2 expression and hormone-sensitive ($P = .66$) or triple-negative tumors ($P = .14$) was detected (Figure 1C). Thus, 96.7% of the HER2+ samples scored positive for CB_2 expression (Figure 1C). Moreover, 65.2% of them expressed elevated levels of CB_2 (scores 2 and 3) (Figure 1C). Importantly, these HER2+/high CB_2 patients had decreased overall survival (hazard ratio [HR] = 0.29, 95% confidence interval [CI] = 0.09 to 0.71, $P = .009$]) (Figure 2A) and higher probability for suffering local recurrence (HR = 0.09, 95% CI = 0.049 to 0.54, $P = .003$) (Figure 2B) and for developing distant metastases (HR = 0.33, 95% CI = 0.13 to 0.75, $P = .009$) (Figure 2C) than HER2+/low CB_2 (scores 0 and 1) patients. Similar observations were made when CB_2 mRNA levels were analyzed in public DNA microarray datasets (HR = 0.25, 95% CI = 0.07 to 1.05, $P = .06$ in Figure 2D; HR = 0.43, 95% CI = 0.18 to 0.94, $P = .04$ in Figure 2E; and HR = 0.52, 95% CI = 0.33 to 0.83, $P = .007$ in Figure 2F) (18–20). Of interest, this association was not observed in HER2- patients (Figures 2G-I; HR = 0.71, 95% CI = 0.30 to 1.56, $P = .36$ in G; HR = 0.83, 95% CI = 0.36 to 1.86, $P = .64$ in H; and HR = 0.48, 95% CI = 0.14 to 1.30, $P = .14$ in I). Together, these results show a strong association between CB_2 expression and tumor aggressiveness in HER2+ breast cancer.

Impact of CB_2 Knock-out on Breast Tumor Generation and Progression

We next analyzed whether there was a cause and effect link between elevated CB_2 expression and increased aggressiveness in HER2+ tumors. First, we observed that CB_2 expression in the noncancerous mammary glands of adult wild-type (WT) female mice was virtually undetectable (Supplementary Figure 1, A and B, available online). Moreover, it remained very low and unchanged during adult mammary gland development (Supplementary Figure 1, A and B, available online). These results suggest that CB_2 may not play a major role in the physiology of the healthy adult mammary gland. Next, we analyzed breast tumor generation and progression in an animal model of HER2-driven breast cancer (the MMTV-neu mouse) in which CB_2 expression was knocked out (Supplementary Figure 2, A and B, available online). MMTV-neu:$CB_2^{-/-}$ mice (n = 42) showed a striking delay in tumor onset as compared with their WT littermates (n = 67) ($P = .03$) (Figure 3A). Upon early detection, tumor histological features were very similar in both groups (low-grade adenocarcinomas with no lymphatic invasion) (Supplementary Figure 2C, available online), the only apparent difference between them being their mitotic index (medium in WT animals and low in the $CB_2^{-/-}$ population) (Supplementary Figure 2C, available online). Forty days after their appearance, 100.0% of the CB_2 KO-derived tumors kept their original histological characteristics, while

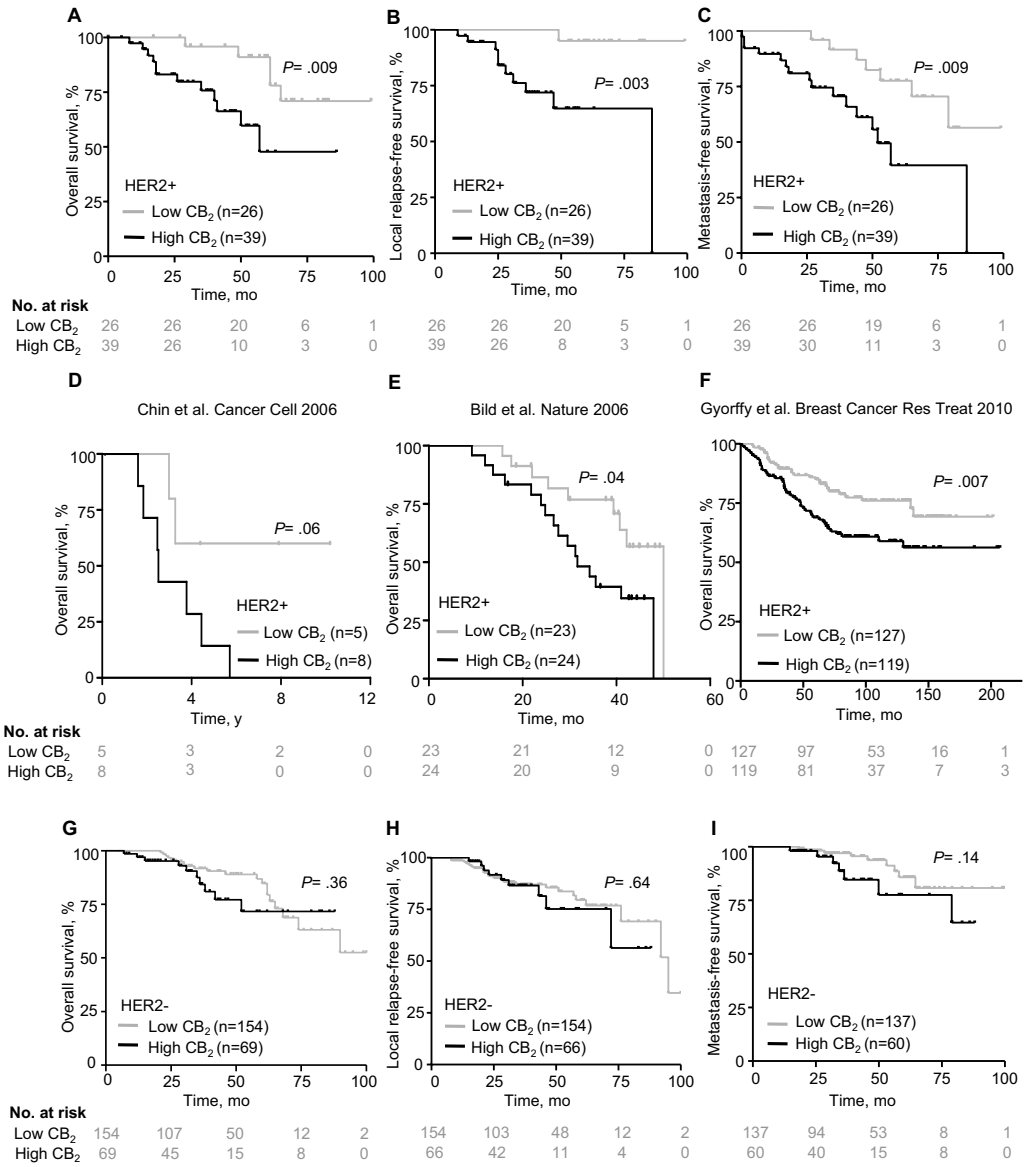

Figure 2. Prognostic relevance of tumor CB_2 expression. Kaplan-Meier curves for overall survival **(A, D-G)**, survival with no recurrence in the breast **(B and H)** and metastasis-free survival **(C and I)**. Numbers below x-axes correspond to the number of patients at risk in each group at the indicated time points. Data plotted in panels **(A-C)** correspond to the 65 human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2)+ samples included in tissue microarray (TMA) # 2 (see Methods). Data plotted in panels **(G-I)** correspond to the 224 HER2- samples included in TMA # 2. Data plotted in panels **(D and E)** were obtained from the microarray data sets published in ArrayExpress database (accession number E-TABM-158) **(D)** and GEO database (accession number GSE3143) **(E)**. Data plotted in **(F)** were obtained from (20) through the Kaplan-Meier Plotter (www.kmplot.com). In **(D-E)**, samples were ranked by CB_2 mRNA expression, and the best cutoff was manually selected. In **(F)**, the best cutoff was automatically selected by the software. Survival curves were statistically compared by the log-rank test. All statistical tests were two-sided.

40.0% of those derived from WT animals were solid carcinomas with necrotic areas (Supplementary Figure 2C, available online). At the final stage of the disease (90 days after tumor appearance), $CB_2^{-/-}$ tumors had negligible changes in their histology (although 40.0% of them presented necrotic areas), but the WT group included 40.0% of solid carcinomas, 40.0% of tumors with necrotic areas, and 20.0% of tumors with evident signs of lymphatic invasion (Supplementary Figure 2C, available online), all of them signs of more aggressive tumors.

The lack of CB_2 receptors also reduced the number of tumors generated per animal (P = .03) (Figure 3B) and slowed down tumor growth (P = .003) (Figure 3C). The delayed tumor onset and the decreased tumor multiplicity and growth associated with the lack of CB_2 receptors were accompanied by reduced levels of cyclin D1 and increased levels of the CDK inhibitor p21 in the tumors (Figure 3, D and E), both of which are hallmarks of HER2-induced malignant transformation and progression (21–23). Additionally, the levels of the endocannabinoid anandamide were higher in $CB_2^{-/-}$-derived tumors than in $CB_2^{+/+}$ tumors (Supplementary Figure 2D, available online), and the mRNA levels of the enzyme responsible for anandamide degradation (FAAH) were lower in CB_2-deficient tumors than in CB_2

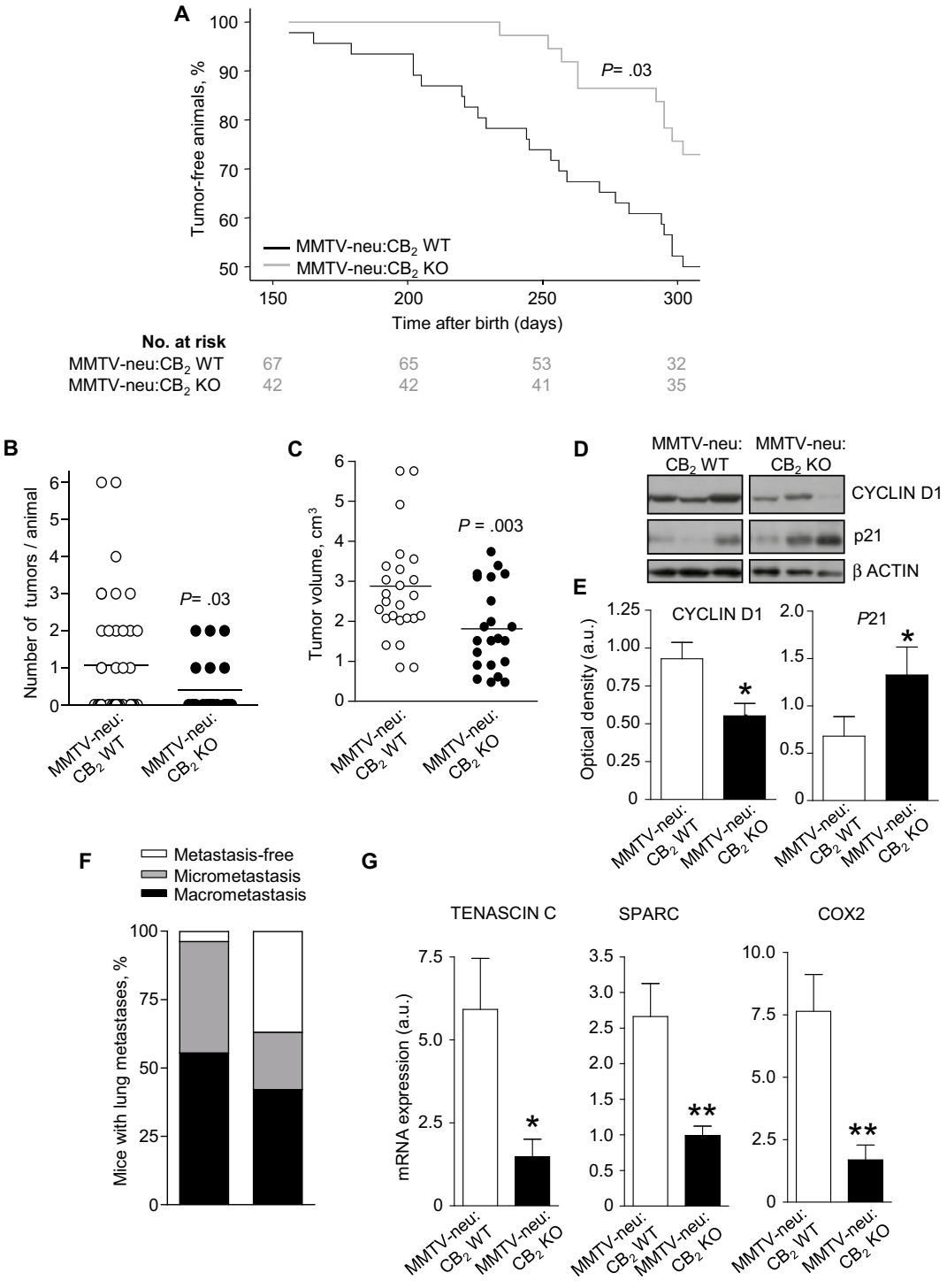

Figure 3. Impact of CB_2 knock-out on breast tumor generation and progression. **A)** Kaplan-Meier curves for tumor onset in MMTV-neu:CB_2 wild-type (WT) and MMTV-neu:CB_2 KO mice. Numbers below x-axes correspond to the number of mice at risk in each group at the indicated time points. Results were analyzed by the log-rank test. **B)** Number of tumors generated per animal 90 days after first tumor arousal. **C)** Tumor volume 70 days after tumor appearance. **D)** Western blot analysis of Cyclin D1 and p21 in tumors generated by the indicated mice. Three representative samples per experimental group are shown. **E)** Densitometric analysis of the levels of the indicated proteins (determined by Western blot; n = 7 for MMTV-neu:CB_2 WT tumors, and n = 6 for MMTV-neu:CB_2 KO tumors). Results are expressed in arbitrary units. **F)** Percentage of animals with lung metastases 90 days after tumor arousal. Lung tumor masses were classified as macrometastases when they were visible to the naked eye at dissection and as micrometastases when they were only detectable by hematoxylin and eosin staining. **G)** mRNA levels (as determined by real-time quantitative polymerase chain reaction) of Tenascin C, SPARC, and COX2 in tumors generated by the indicated mice. Results are expressed in arbitrary units (n = 16 for MMTV-neu:CB_2 WT tumors and n = 16 for MMTV-neu:CB_2 KO tumors). Except in A, data were analyzed by analysis of variance with a post hoc analysis by the Student-Newman-Keuls' test. * $P < .05$; ** $P < .01$ vs MMTV-neu:CB_2 WT mice. All statistical tests were two-sided.

WT lesions (Supplementary Figure 2E, available online). Finally, CB_2 deficiency produced a remarkable reduction in the percentage of animals with lung metastases (Figure 3F). This phenotype associated with decreased tumor levels of tenascin-C, SPARC, and COX2 (Figure 3G), which have been proposed to mediate metastasis specifically directed to the lungs (24). Together, these results indicate that CB_2 plays an important role in promoting HER2+ breast tumor generation and progression.

Mechanistic Insight into the Association Between CB_2 and HER2 Expression

Because we observed that virtually all HER2+ human tumors express CB_2 (Figure 1), we analyzed whether HER2 controls the expression of the cannabinoid receptor. Ectopic overexpression of HER2 in triple-negative (no estrogen receptor, progesterone receptor, and HER2 expression) MDA-MB-231 cells resulted in an increased transcription of CB_2 (Figure 4A). The in silico analysis of the CB_2 promoter sequence revealed the existence of, among others, several ELK1-binding sites (25) (Supplementary Figure 3A, available online). ELK1 belongs to the ETS transcription factor family, which has been related to cancer (26) and is a well-established target of the ERK cascade (27). We observed that HER2 overexpression activated ELK1, an effect that was accompanied by the activation of ERK (Figure 4B). Of interest, incubation with the MEK inhibitor U0126 prevented the enhancement of p-ELK1 levels (Figure 4B). Moreover, pharmacological inhibition of MEK (Figure 4C) and genetic knock-down of ELK1 (Figure 4D; Supplementary Figure 3B, available online) blocked the increase in CB_2 mRNA levels elicited by HER2 overexpression. Likewise, ELK1 knock-down (Supplementary Figure 3C, available online) decreased CB_2 mRNA levels in breast cancer cells that endogenously overexpress HER2, an effect that was not observed in HER2-negative cells (Figure 4E). By chromatin immunoprecitation assays, we confirmed that ELK1 physically interacts with the CB_2 promoter and that this interaction is enhanced upon HER2 overexpression and is prevented by inhibition of the ERK cascade (Figure 4F). Moreover, ELK1 was able to activate the CB_2 promoter. Thus, transfection of HEK293T cells with a luciferase reporter encoding the CB_2 gene promoter (pGL3- CB_2) together with a constitutively active ELK1-expressing plasmid resulted in an increased luciferase activity when compared with cells transfected with pGL3- CB_2 only (Figure 4G). Point mutations in the CB_2 promoter revealed that the putative ELK1-binding sites located at positions -71 and -89 are the ones responsible for ELK1-induced activation of CB_2 expression (Figure 4G). Together, these observations demonstrate that HER2 promotes CB_2 upregulation by activating the transcription factor ELK1 via ERK activation. Supporting the relevance of this observation, the analysis of 1453 human breast cancer samples from seven different public DNA microarrays (19,28–33) showed a strong association ($P < .001$) between ELK1 and CB_2 mRNA expression (Figure 4, H and I), and the immunofluorescence analysis of HER2+ breast cancer biopsies revealed that CB_2-positive cancer cells presented nuclear ELK1 immunoreactivity (Figure 4J).

Analysis of the Potential Molecular Interaction Between HER2 and CB_2

We wanted to determine whether the HER2-CB_2 functional crosstalk was the result of a molecular interaction between the receptors. Immunofluorescence analysis of human HER2+ breast cancer cells revealed that the two receptors colocalize (Figure 5A). Moreover, immunoprecipitation of HER2 in HEK cells produced the coprecipitation of CB_2, and vice versa (Figure 5B). The HER2-CB_2 molecular association in cancer cells was confirmed using the proximity ligation assay. Thus, ectopic overexpression of HER2 in MDA-MB-231 cells enhanced the levels of CB_2 (Figure 5C), and this effect was accompanied by the appearance of fluorescent dots, ie, HER2-CB_2 heteromers (Figure 5D). This fluorescent signal was not evident either in cells that do not express HER2 (with very low levels of CB_2) or in cells in which CB_2 expression was knocked down by means of selective shRNA (Figure 5D). Importantly, the presence of HER2/CB_2 heterodimers was detected in human breast cancer cells that endogenously overexpress HER2 (Figure 5E) and in HER2-positive human breast cancer tissue (Figure 5F). Again, the heteromer fluorescent signal was not evident either when CB_2 was stably silenced in cells (Figure 5E) or in tumors that do not overexpress HER2 (Figure 5F). These results confirm that CB_2 is upregulated by HER2 and support that HER2 and CB_2 form heteromers in cancer cells.

Role of CB_2 in the Control of the Pro-oncogenic Features of HER2+ Breast Cancer Cells

We next analyzed the biological consequences of the HER2-induced CB_2 upregulation. HER2 overexpression in MDA-MB-231 cells (which effectively enhanced CB_2 levels) (Figure 5C) increased cancer cell viability (Figure 6A) and stimulated properties of cancer cells intimately related to tumor progression, ie, invasion (Figure 6B) and anchorage-independent growth (Figure 6C). These effects were prevented by CB_2 knock-down (Figure 6, A-C). Likewise, genetic silencing of CB_2 (Supplementary Figure 3D, available online) reduced cell viability (Figure 6D), cell invasion (Figure 6E), colony formation in soft agar (Figure 6F), and the expression of metastasis markers (Supplementary Figure 3E, available online) in cells endogenously overexpressing HER2, an effect that was not observed in HER2-negative cells. Moreover, the growth of MDA-MB-231 HER2-overexpressing orthotopic xenografts (Figure 6G) and the generation of lung metastases (Figures 6, H and I; Supplementary Figure 3F, available online) were statistically significantly impaired upon stable CB_2 knock-down (Supplementary Figure 3G, available online). Similarly, tumors generated from HER2-amplified cells statistically significantly reduced their growth when CB_2 was silenced (Figure 6J). Collectively, these data show that CB_2 promotes pro-oncogenic responses in a HER2+ context.

Potential Targets of CB_2-HER2 Pro-oncogenic Signaling

Next, we wanted to identify CB_2 targets responsible for its protumoral activity. Upon modulation of CB_2 and HER2 expression and by means of a phospho-kinase array, we detected substantial alterations in some members of the SRC family of nonreceptor tyrosine kinases, which has been extensively related to cancer (34–36). Specifically, we found that the expression of phosphorylated LYN, LCK, YES, FGR, HCK, and FYN decreased upon CB_2 knock-down (Supplementary Figure 4, A and B, available online). However, this effect was observed both in HER2+ and HER2- cells (Supplementary Figure 4, A and B, available online), which suggests that, although these may be relevant CB_2 targets in breast cancer, they are not HER2+ context specific. Of interest, the phosphorylated form of another member of the SRC family (c-SRC), which has particular relevance in cancer development and progression (34), was specifically upregulated upon HER2

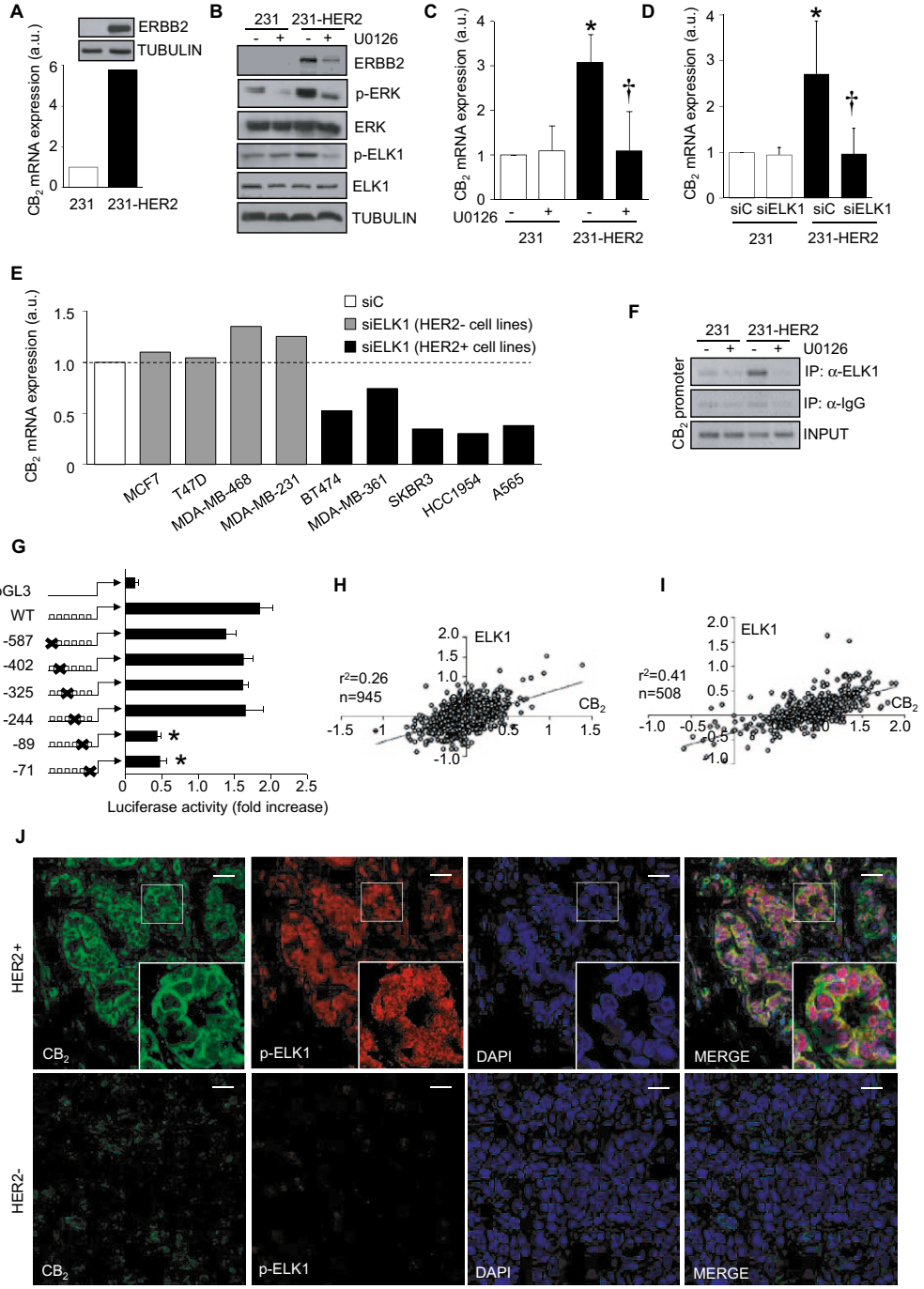

Figure 4. Mechanistic insight into the association between CB_2 and human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2) expression. **A)** ERBB2/HER2 protein expression (**upper panel**) and CB_2 mRNA expression (**lower panel**) in MDA-MB-231-HER2 (231-HER2) and MDA-MB-231 cells (231). **B)** Western blot analysis of the indicated proteins, in the presence or in the absence of the MEK inhibitor U0126 (5 μM). **C-D)** CB_2 mRNA expression (in arbitrary units), in the presence/absence of U0126 **(C)**, or after transfection with ELK1 siRNAs (siELK1) or with a nontargeted siRNA (siC) **(D)** (n = 3 independent experiments). **E)** Effect of ELK1 knockdown on CB_2 mRNA expression in different human breast cancer cells endogenously overexpressing (**black bars**) or not (**gray bars**) HER2. Results are expressed in arbitrary units vs mRNA expression in the corresponding cells transfected with a control siRNA (siC), which was set at 1 in all cases (**white bar**). **F)** ChIP assay in cells treated with or without U0126. Immunoprecipitation was performed with an anti-ELK1 Ab (or a nonspecific rabbit IgG as control). **G)** CB_2 gene promoter activity as determined by a luciferase reporter (n = 3 independent experiments). Drawings (**left**) represent the CB_2 promoter construct transfected in each case. A constitutively active ELK1-containing plasmid was always cotransfected with the CB_2 promoter. Line 1, CB_2 promoter empty vector. **H and I)** Correlation of CB_2 and ELK1 expression (analyzed by the Pearson's correlation test) in human breast cancer samples from seven public DNA microarrays ([19,24,28,30,31,33] in **[H]** and [31] in **[I]**). **J)** Immunofluorescence analysis of CB_2 (**green**) and phospho-ELK1 (**red**) in a HER2-positive (**upper panels**) and a HER2-negative (**lower panels**) human breast cancer sample. Cell nuclei are stained in **blue**. **Scale bar** = 100 μm. Except in **(I and J)**, data were analyzed by analysis of variance with a post hoc analysis by the Student-Newman-Keuls' test. * $P < .01$ vs vehicle-treated **(C)** or siC-transfected **(D)** 231 cells; † $P < .05$ vs vehicle-treated **(C)** or siC-transfected **(D)** 231-HER2 cells; * $P < .01$ vs WT **(G)**. All statistical

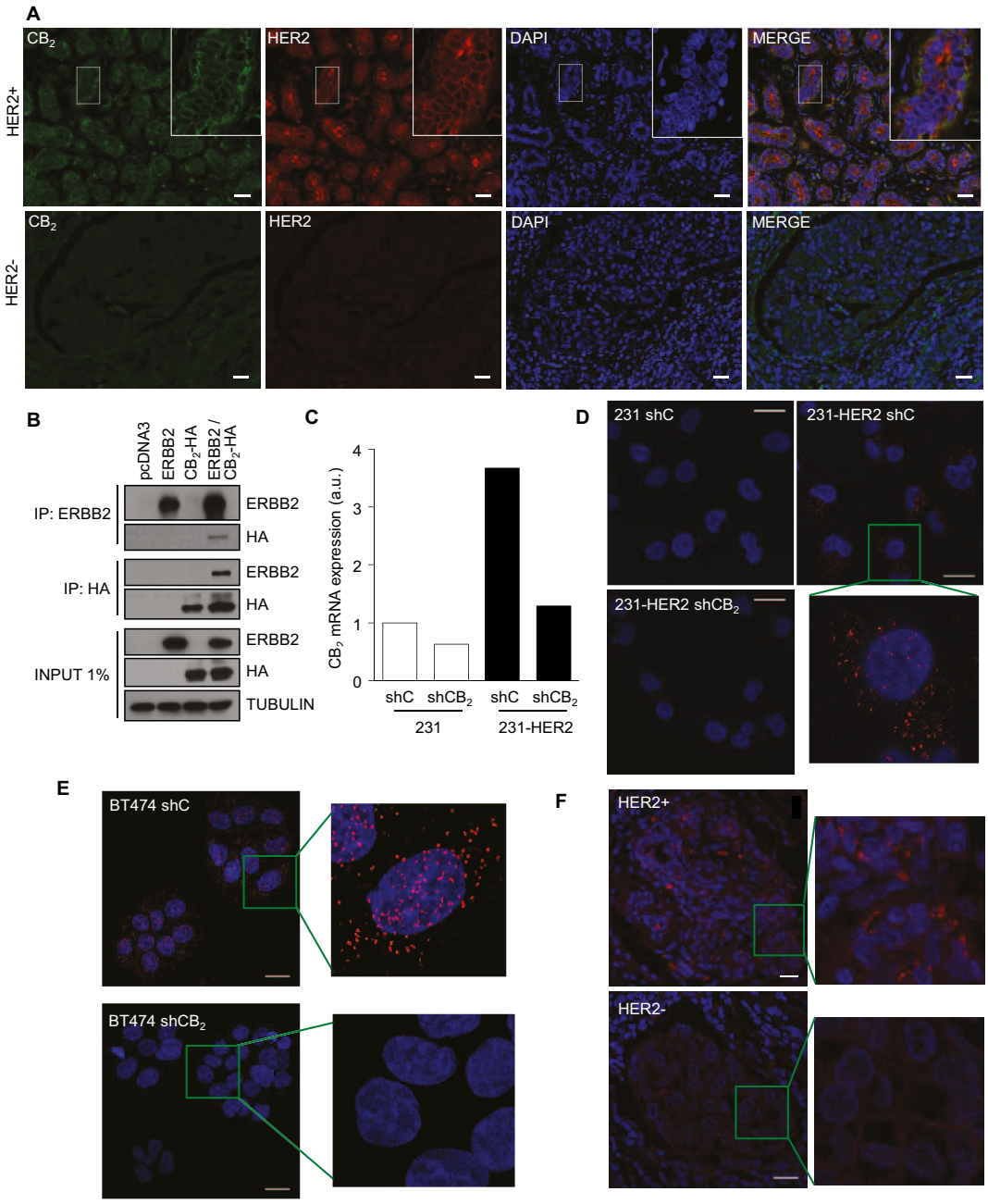

Figure 5. Analysis of the potential molecular interaction between HER2 and CB_2. **A)** Immunofluorescence analysis of CB_2 (**green**) and human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2) (**red**) protein expression in a HER2-positive (**upper panels**) and a HER2-negative (**lower panels**) human breast tumor sample. Cell nuclei are stained in **blue**. **Scale bar** = 100 μm. **B)** Analysis of the HER2-CB_2 molecular interaction by immunoprecipitation assay in HEK cells transfected with an ERBB2 overexpression plasmid, a HA-tagged CB_2 plasmid, both of them simultaneously or the corresponding empty vector (pcDNA3). **C)** CB_2 mRNA expression, as determined by real-time quantitative polymerase chain reaction and expressed in arbitrary units, in MDA-MB-231 cells (231) or 231 cells stably overexpressing HER2 (231-HER2), upon stable knock-down of CB_2 with a selective shRNA ($shCB_2$) pool or after infection with lentiviral particles containing a scrambled shRNA (shC). **D-F)** Proximity ligation assays in 231 shC cells and in 231-HER2 cells infected with either shC or $shCB_2$ particles **(D)**, in BT474 shC and BT474 $shCB_2$ cells **(E)**, and in a HER2-negative and a HER2-positive human breast cancer sample **(F)**. Cell nuclei are stained in **blue**, and the **red** fluorescent signal corresponds with CB_2/HER2 heteromers. **Scale bars** = 20 μm.

overexpression and downregulated by CB_2 knock-down in that high-HER2 context (Figure 7A; Supplementary Figure 4B, available online). Moreover, when CB_2 expression was restored in MDA-MB-231-HER2 $shCB_2$ cells, p-c-SRC levels were increased, an effect that was not evident in the MDA-MB-231 that do not overexpress HER2 (Figure 7B). Likewise, a decrease in p-c-SRC upon CB_2 silencing was observed in a panel of five breast cancer cell lines that endogenously overexpress HER2 (Figure 7C).

Next, we tested whether c-SRC was responsible for CB_2-driven oncogenesis. First, we observed that mouse NIH/3T3 embryonic

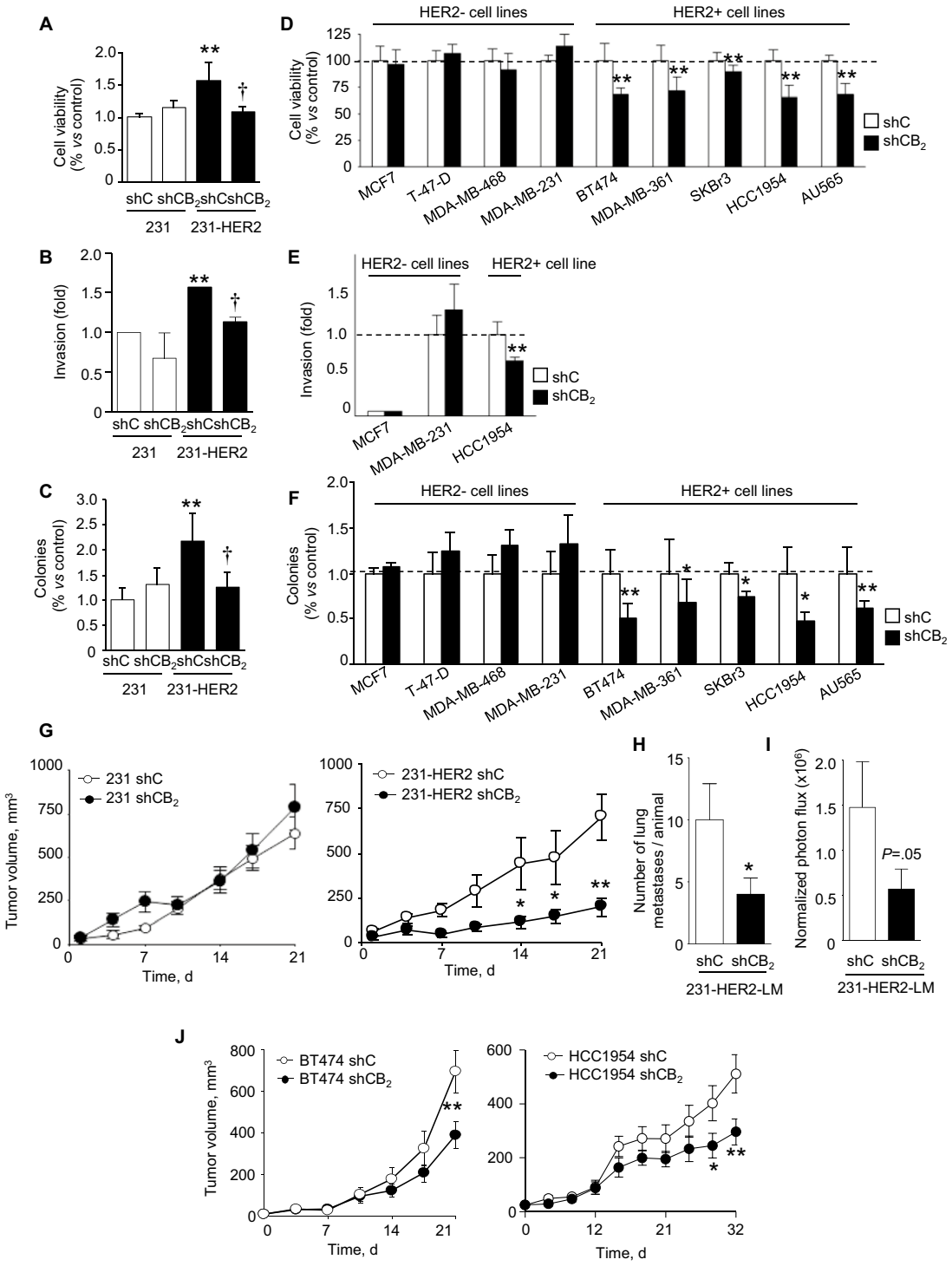

Figure 6. Role of CB_2 in the control of the pro-oncogenic features of HER2+ breast cancer cells. **A-F)** Cell viability as determined by the MTT test **(A and D)**, invasion in matrigel-coated Boyden chambers **(B and E)**, and number of colonies generated in soft agar **(C and F)** of MDA-MB-231 (231) and MDA-MB-231-HER2 cells (231-HER2) **(A and B)** or of the indicated HER2-positive or HER2-negative cell lines **(D-F)** stably expressing a shRNA selectively targeting CB_2 ($shCB_2$) or a scrambled shRNA (shC). **G and J)** Evolution of tumor volume in mice orthotopically injected with either 231 or 231-HER2 cells stably expressing $shCB_2$ or shC **(G)**, or with the indicated HER2-amplified cells stably expressing the same shRNAs **(J)**. **H)** Evaluation of the number of lung metastases generated per animal by injection of lung-seeking MDA-MB-231-HER2 cells (231-HER2-LM) stably expressing $shCB_2$ or an shC into the mouse lateral tail vein. **I)** Quantification of the lung bioluminescence signal in the two experimental groups. Data were analyzed by analysis of variance with a post hoc analysis by the Student-Newman-Keuls' test ($n \geq 3$ independent experiments, except in **[G-J]**). * $P < .05$ and ** $P < .01$ vs the corresponding shC cell line; † $P < .01$ vs shC-231-HER2 cells. All statistical tests were two-sided.

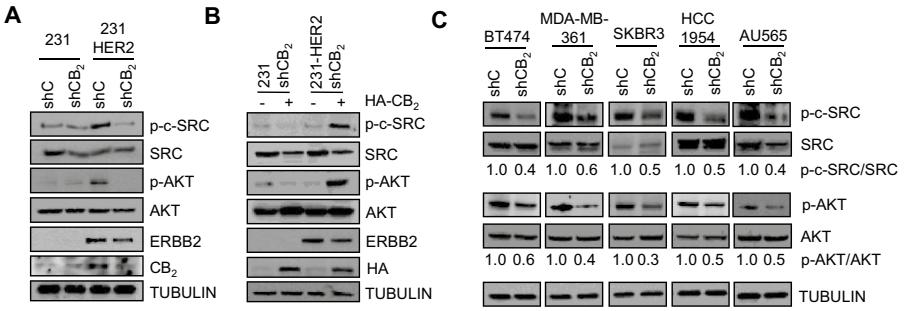

Figure 7. Involvement of c-SRC in CB_2-induced human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2)–mediated pro-oncogenic signaling. **A-C)** Western blot analysis of the indicated proteins in MDA-MB-231 (231) and MDA-MB-231-HER2 (231-HER2) cells stably expressing a shRNA selectively targeting CB_2 ($shCB_2$) or a scrambled shRNA (shC) **(A)**, or in 231 $shCB_2$ or 231-HER2 $shCB_2$ cells stably expressing a HA tagged-CB_2 plasmid (HA-CB_2) or the corresponding empty vector (pcDNA3) **(B)**, or in the indicated HER2-amplified cells stably expressing $shCB_2$ or shC **(C)**. Numbers in **(C)** correspond to the densitometric analysis of the respective bands.

fibroblasts acquire clonogenic properties upon overexpression of either CB_2 or HER2 (Figure 8, A and B). Moreover, the ability of these cells to form colonies in soft agar statistically significantly increased when the two receptors were simultaneously overexpressed (Figure 8, A and B). Disruption of c-SRC signaling by using a c-SRC-dominant negative construct prevented the oncogenic phenotype induced by CB_2 plus HER2 (Figure 8, A and B). Of interest, while the HER2-mediated increased clonogenicity was prevented by blocking c-SRC signaling, the CB_2-induced clonogenic response was not (Figure 8, A and B), which indicates that CB_2 promotes c-SRC activation (and the subsequent clonogenic response) via HER2. We then performed colony formation experiments with human HER2-amplified breast cancer cells. Specifically, we overexpressed CB_2 in five HER2+ cell lines and observed an increase in the levels of p-c-SRC (Figure 8C). As expected, this increase in activated c-SRC was accompanied by an enhanced clonogenicity (Figure 8D). Importantly, pharmacological inhibition of c-SRC with Saracatinib (a SRC family/Abl dual-kinase inhibitor) in CB2-overexpressing cells kept both clonogenicity (Figure 8D) and p-c-SRC expression (Figure 8C) at the same level as in pcDNA3-transfected cells treated with the inhibitor, which further suggests that CB_2-driven oncogenesis is mediated by c-SRC activation. Finally, and in further support of a causal link between the CB_2/HER2/c-SRC axis and pro-oncogenic events, we found a decreased c-SRC and AKT activation in tumors generated by CB_2-deficient animals (which present a less aggressive phenotype [Figure 3]) with respect to their WT littermates (Figure 8, E and F), and the analysis of human tumor biopsies revealed that HER2+ breast cancer cells expressing activated c-SRC also expressed CB_2 (Figure 8G).

Discussion

Here we demonstrate not only that the cannabinoid receptor CB_2 exerts a remarkable pro-oncogenic function in HER2+ breast cancer but also that CB_2 plays a pivotal role in HER2-mediated pro-oncogenic signaling (Figure 8H). It is widely accepted that GPCRs and TKRs control critical biological processes intimately related to oncogenesis and that the functional cross-talk between members of these two receptor superfamilies (eg, transactivation of TKRs by GPCR-mediated signaling) may have important consequences in the progression and resistance to TKR-targeted therapies of some types of cancer (37,38), including HER2+ breast cancer (39). In some cases, the functional cross-talk between GPCRs and TKRs might rely on a physical interaction between receptors. Regarding HER2 specifically, it has been reported that this receptor can form a complex with the β2-adrenergic receptor in the heart and brain, which is required for mitogen-activated protein kinase activation induced by multiple GPCR agonists in cardiac myocytes (40). Here we show for the first time that a TKR (HER2) forms heteromers with a GPCR (CB_2) in cancer cells. These findings reveal an unprecedented mechanism of control of HER2 activity that involves cannabinoid receptor CB_2, and they suggest that the simultaneous targeting of the two receptors (or common downstream effectors) may be a reasonable therapeutic strategy. Because dual-targeting approaches are showing positive results in preclinical and clinical contexts when the targets are different members of the ERBB family (mainly ERBB1 and ERBB2) or even different domains of the same receptor (neutralizing antibodies + tyrosine kinase inhibitors, for example) (41), it is tempting to speculate that the combination of anti-HER2 compounds with cannabinoids targeting CB_2 may have synergistic antitumoral effects. Interestingly, there is evidence showing that, at least at the preclinical level, the combination of cannabinoids with other anticancer therapies results in improved responses when compared with the corresponding individual treatments (11,42–45). It would therefore be desirable that future clinical trials determine whether these preclinical findings can be extrapolated to the cancer patient.

Our findings also show that the nonreceptor tyrosine kinase c-SRC plays a pivotal role in CB_2-induced HER2 pro-oncogenic signaling (Figure 7). c-SRC promotes cell proliferation, survival, migration, and angiogenesis (34), and its deregulation is associated with oncogenesis (35,36) and poor patient prognosis (46). Together, these features made c-SRC an excellent target for the clinical development of specific inhibitors (35,36,46,47). In HER2+ breast cancer in particular, c-SRC activation has been implicated in the generation of brain metastases (48), a condition that has no curative treatment, and in the development of trastuzumab resistance (49). Interestingly, treatment of brain metastasis–bearing mice with a combination of Lapatinib (which targets ERBB1 and ERBB2) and a c-SRC inhibitor slowed down the growth of the metastases (48), and treatment of trastuzumab-resistant cells/tumors with a c-SRC inhibitor restored trastuzumab response (49). These data suggest that blocking c-SRC may be an effective manner to treat two important remaining clinical challenges in HER2+ breast cancer: the management of highly metastatic tumors (especially those colonizing the central nervous system) and trastuzumab resistance (both innate and acquired). Nonetheless, additional experiments should be performed to analyze the involvement of CB_2 in c-SRC-mediated trastuzumab resistance and generation of brain metastases.

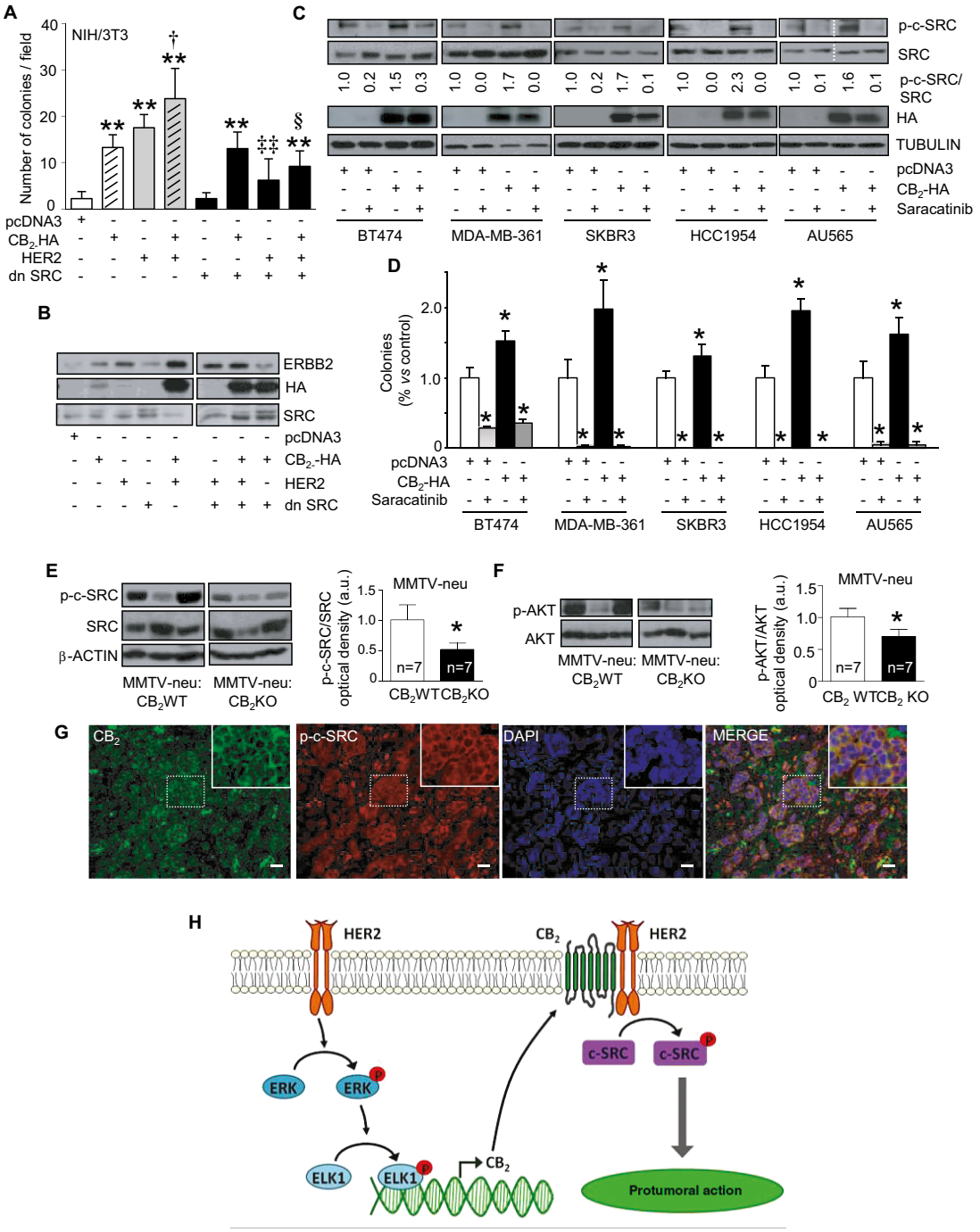

Figure 8. Involvement of c-SRC in CB_2-induced human V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2 (HER2)–mediated pro-oncogenic signaling. **A-D)** Anchorage-independent growth (**[A and D]**, n = 3 independent experiments) and expression of ERBB2, HA-CB_2 and the indicated proteins **(B and C)** (as determined by Western blot), in NIH/3T3 fibroblasts **(A and B)** and in a panel of five HER2+ human breast cancer cell lines **(C and D)** transiently transfected with the indicated constructs and/or incubated with 1 μM Saracatinib. In **(B and C)** (AU565 cells), lanes were run on the same gel but were noncontiguous. **E and F)** Western blot (**left panels**) and densitometric analysis (**right panels**) of phospho-c-SRC **(E)** and phosphor-AKT **(F)** in breast tumors generated by MMTV-neu:CB_2 WT and MMTV-neu:CB_2 KO mice. **G)** Immunofluorescence analysis of CB_2 (**green**) and phospho-c-SRC (**red**) in a human HER2+ breast tumor sample. Cell nuclei are stained in **blue**. **Scale bar** = 100 μm. **H)** Proposed role of CB_2 in HER2-driven pro-oncogenic signaling: HER2 enhances CB_2 expression by activating the transcription factor ELK1 via ERK. Increased CB_2 expression promotes HER2 pro-oncogenic signaling by activating the tyrosine kinase c-SRC. Data were analyzed by analysis of variance with a post hoc analysis by the Student-Newman-Keuls' test. * $P < .05$ and ** $P < .01$ vs pcDNA3; † $P < .05$ vs CB_2 or HER2; ‡ $P < .01$ vs HER2; § $P < .05$ vs HER2-CB_2 **(A and D)**. * $P < .05$ vs MMTV-neu:CB_2 WT mice **(E and F)**. All statistical tests were two-sided.

Our results clearly reveal a pro-oncogenic role of CB_2 in HER2+ breast cancer. However, it has been widely described that pharmacological activation of this particular receptor exerts antitumoral effects in different models of breast (14,16,17,50,51) and many other types of cancer (11). Further experiments should be performed to get a deeper insight into the molecular details of this bimodal effect of CB_2 receptor functionality. For example, it would be interesting to know whether different cannabinoid stimuli (ie, an endogenous tone vs an exogenous pharmacological activation) produce a different activation of CB_2/HER2-mediated signaling in terms of intensity and/or specific pathways.

Finally, we have observed a strong association between higher CB_2 protein expression in HER2+ breast tumors and lower patient overall relapse-free and metastasis-free survival. It has been previously shown that the levels of CB_2 are elevated in breast (16,17,51) and many other types of cancer compared with healthy matching tissue (11) and in the more aggressive (high-grade) breast (16,17) and brain (52–54) tumors compared with the respective less aggressive (low grade) tumors. Recently, an association between CB_2 expression and overall and disease-free survival of patients with squamous cell carcinoma of the head and neck has also been reported (55). All these observations may serve to exploit CB_2 as a new prognostic marker in oncology (at least in certain types of tumors).

In conclusion, here we present solid insight into an unprecedented pro-oncogenic effect of the CB_2-HER2 signaling axis. However, we acknowledge some limitations in our study. First, the cell and animal models used in this study are well validated on translational grounds, but they do not fully recapitulate the pathology found in the actual HER2+ breast cancer patient. In this respect, although we have been able to unravel a strong association between CB_2 expression in human HER2+ tumor specimens and the prognosis of the donor patients, it would be desirable that this association be established in larger patient populations to further support the potential impact of our observations on therapeutic decision-making. This should be ideally accompanied by clinical studies aimed at evaluating the safety and efficacy of strategies targeting, for example, CB_2 and SRC, in combination with widely accepted anti-HER2+ breast cancer chemotherapies and immunotherapies. Additionally, although our findings support the existence of HER2-CB_2 heteromers, further studies should be performed to unveil the functional relevance of these complexes in human breast cancer and whether—and, if so, how—they actually drive CB_2/HER2-evoked signaling at different stages of tumor malignancy. Notwithstanding such limitations, our study provides the first proof of concept on the action of CB_2 as a new key player in HER2+ breast cancer biology.

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Notes

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Over every mountain there is a path, although it may not be seen from the valley.

Theodore Roethke